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Molecular cloning and tissue expression analyses of two novel pepper genes: heterotrimeric G protein beta 2 subunit and ArcA1

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ABSTRACT. We isolated two transcription factor genes, heterotrimeric G protein beta 2 subunit (Gβ2) and ArcA1, from pepper (*Capsicum annuum*). The complete coding sequences were amplified using reversed transcriptase PCR based on conserved sequence information of *Solanum lycopersicum* and several other plant species. Nucleotide sequence analysis of these two genes revealed that the pepper Gβ2 gene encodes a protein of 376 amino acids that belongs to the WD40 superfamily. Tissue expression analysis indicated that this gene is highly expressed in the pericarp, moderately expressed in stem, flower, placenta, and leaves, and weakly expressed in seed. There was no expression in the roots. The ArcA1 gene encodes a protein of 331 amino acids that also belongs to the WD40 superfamily. Tissue expression analysis indicated that the pepper ArcA1 gene is moderately expressed in the pericarp and weakly expressed in seed. There was no expression in root, stem, flower, placenta, or leaves.

Key words: *Capsicum annuum* L.; Heterotrimeric G protein beta 2 subunit; ArcA1; Gene expression profile

INTRODUCTION

Pepper (*Capsicum annuum* L.) is a dicotyledonous flowering member of the Solanaceae, which includes potato, tomato, eggplant, and African eggplants (Hunziker, 2001; Knapp, 2002). The plant has been cultivated in the Yunnan Province of China for many years. The Yunnan purple pepper No. 1 (YNPP1) is a species in that the whole plant is violet black, especially the fruit. Shades ranging from violet to black in *C. annuum* L. are attributed to anthocyanin accumulation.

G proteins (guanine nucleotide-binding proteins) transmit extracellular chemical signals, causing intracellular changes. They communicate signals from hormones, neurotransmitters, and other signaling factors (Reece and Campbell, 2002). The term G protein can refer to 1 of 2 distinct protein families. Heterotrimeric G proteins, sometimes referred to as the "large" G proteins, are activated by G protein-coupled receptors and are composed of alpha (α), beta (β), and gamma (γ) subunits (Hurowitz et al., 2000). Different types of heterotrimeric G proteins share a common mechanism. They are activated in response to a conformation change in the G protein-coupled receptor, exchange GDP for GTP, and dissociate to activate other proteins in the signal transduction pathway. The specific mechanisms, however, differ between types.

ArcA1 contains 7 WD40 repeats and was first isolated from *Solanum lycopersicum* (Kiyosue and Ryan, 1999). ArcA, a member of the G protein beta-subunit family, was isolated from tobacco BY-2 cells as an auxin-responsive gene. ArcA was characterized, with emphasis on expression, to elucidate the function of the gene product in plant cells. Accumulation of ArcA message was detected only after treatment with auxins and not after treatments with other phytohormones or $CdCl_2$, suggesting that ArcA responsiveness is exclusive to auxin. The putative ArcA promoter region was fused to a beta-glucuronidase (GUS) reporter and transient expression was analyzed in tobacco BY-2 cells (Ishida et al., 1996). The possibility that ArcA gene products induce cell division was discussed (Ishida et al., 1993).

In this study, the novel regulatory genes, Gβ2 and ArcA1, were isolated from YNPP1. Their expression patterns provide a foundation for further understanding of the biochemical functions of Gβ2 and ArcA1 in *C. annuum* L.

MATERIAL AND METHODS

Sample collection

All plants were derived from the College of Horticulture and Landscape, Yunnan Agricultural University. YNPP1 tissues (root, stem, leaf, blossom, pericarp, placenta, and seed) were instantly frozen in liquid nitrogen and stored at -80°C before use.

Total RNA extraction and first-strand cDNA synthesis

Total RNA from YNPP1 was extracted with Trizol (TaKaRa) and cDNA was synthesized with the High Fidelity PrimeScript RT-PCR Kit (TaKaRa) according to the manufacturer protocol.

PCR amplification

PCR was performed to isolate the YNPP1 genes from pooled cDNAs from different tis-

sues. Reactions were performed as described previously (Deng et al., 2011). The PCR program of the genes Gβ2 and ArcA1 starts at 94°C for 3 min, followed by 34 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and then an extension at 72°C for 10 min, followed by 4°C hold.

The mRNA and amino acid sequences for Gβ2 and ArcA1 from various plant species archived at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) were used to locate conserved regions by multiple sequence alignment with CLUSTALW 1.8. The following primers were designed: Gβ2-F: 5'-TGAAATGGGATTGGAC-3', Gβ2-R: 5'-CATATGGCCGCTACAC-3'; ArcA1-F: 5'-CGGATATTGCCCAGTC-3', ArcA1-R: 5'-AAC CACGAGATACAAGGAGA-3'.

RT-PCR expression profile

YNPP1 Gβ2 and ArcA1 RT-PCR primers for expression profile analysis were the same as those used for PCR. PCRs were optimized to ensure sufficient product intensity within the linear phase of amplification.

Bioinformatics

PCR amplification was repeated 5 times. Amplification products were cloned into pMD18-T (TaKaRa) and bidirectionally sequenced. At least 10 independent clones were sequenced for each PCR product. Sequencing data were edited and aligned in DNASTAR (DNAStar Inc., USA). cDNA sequences were predicted with GenScan (http://genes.mit.edu/GENSCAN.html). Putative protein theoretical molecular weight (Mw) and isoelectric point (pI), signal peptide, subcellular localization, membrane-spanning regions, and PFAM domains were identified with the Compute pI/Mw Tool (http://us.expasy.org/tools/pi_tool.html), SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/), PSort (http://psort.hgc.jp/), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), and SMART (http://smart.embl-heidelberg.de). The BLASTp Program and Conserved Domain Architecture Retrieval Tool were used to search for similar proteins and conserved domains (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of the nucleotide sequences and deduced amino acid sequences was performed with ClusterX, and phylogenetic trees were constructed in ClustalX and Mega 4.0 with standard parameters. Secondary structures of deduced amino acid sequences were predicted in SOPMA (http://npsa-pbil.ibcp.fr/). The 3-D structures were predicted based on existing 3-D structures derived from amino acid homology modeling on Swiss Server (http://swissmodel.expasy.org/).

RESULTS

RT-PCR of YNPP1 Gβ2 and ArcA1

RT-PCR with pooled tissue cDNAs for YNPP1 Gβ2 and ArcA1 genes yielded products of 1139 and 993 bp (Figure 1).

cDNA nucleotide sequence analysis revealed that the genes are not homologous to any known pepper genes. Sequence prediction showed that the 1139- and 993-bp cDNAs represent 2 genes, which encode 376- and 331-amino acid proteins, respectively.

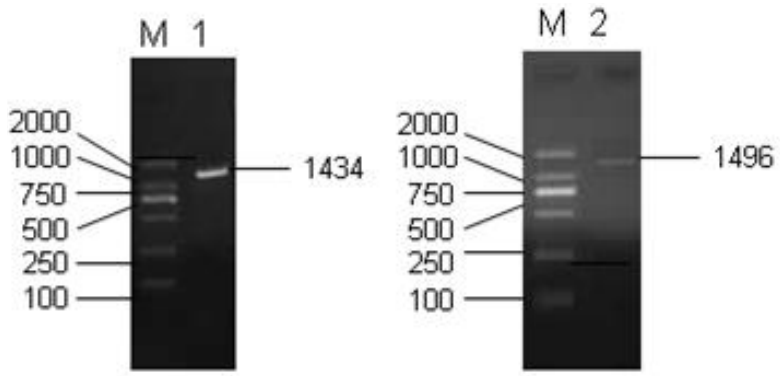

Figure 1. RT-PCR of pepper Gβ2 and ArcA1. *Lane M* = DL2000 DNA marker; *lane 1* = PCR product for Gβ2; *lane 2* = PCR product for ArcA1.

Protein properties

pI and Mw of the deduced proteins were computed. pIs are 6.48 and 5.08 and Mw of the putative proteins are 40,796 and 82,376 Da.

The complete coding sequences (CDS) and the encoded amino acids are presented in Figures 2 and 3.

```
ATGTCAGTGACAGAGCTGAAAGAGCGGcAtATGGTCGCTaCTcAGACTGTAAaTGATCTCCGTGAAAAACTTAAGCAGAAACGT
 M  S  V  T  E  L  K  E  R  H  M  V  A  T  Q  T  V  N  D  L  R  E  K  L  K  Q  K  R
CTTCAATTACTCGACACTGATGTTGCTGGGTATGCAAGGTCACAAGGTAAAACTCCGGTTACCTTtGGCCCAACAGATCTGGTT
 L  Q  L  L  D  T  D  V  A  G  Y  A  R  S  Q  G  K  T  P  V  T  F  G  P  T  D  L  V
TGTTGTAGGATCCTGCAAGGACACACAGGAAAGGTCTATTCACTGGACTGGACTCCTGAAAAAAATCGTATAGTCAGTGCATCC
 C  C  R  I  L  Q  G  H  T  G  K  V  Y  S  L  D  W  T  P  E  K  N  R  I  V  S  A  S
CAAGATGGTAGATTAATAGTGTGGAATGCTCTCACAAGCCAAAAAACCCATGCAATTAAGCTTCCATGTGCTTGGGTTATGACC
 Q  D  G  R  L  I  V  W  N  A  L  T  S  Q  K  T  H  A  I  K  L  P  C  A  W  V  M  T
TGCGCCTTCTCTCCTAGCGGACAGTCTGTTGCTTGTGGTGGCCTTGACAGTGCCTGCTCTATCTTCAACTTAAATTCACCGATC
 C  A  F  S  P  S  G  Q  S  V  A  C  G  G  L  D  S  A  C  S  I  F  N  L  N  S  P  I
GATAAGGATGGGAACCATCCAGTATCGAGAATGCTTAGTGGGCATAAGGGGTATGTGTCATCGTGTCAGTATGTTCCAGATGAG
 D  K  D  G  N  H  P  V  S  R  M  L  S  G  H  K  G  Y  V  S  S  C  Q  Y  V  P  D  E
GATACTCACCTAATAACTAGTTCTGGCGATCAAACATGTGTACTTTGGGATATAACTACTGGCCTAAGAACTTCTGTGTTTGGA
 D  T  H  L  I  T  S  S  G  D  Q  T  C  V  L  W  D  I  T  T  G  L  R  T  S  V  F  G
GGTGAGTTTCAATCTGGGCACACTGCAGATGTATTAAGTGTCTCAATTAGTTCATCTAACCCCAGACTGTTTGTGTCTGGGTCC
 G  E  F  Q  S  G  H  T  A  D  V  L  S  V  S  I  S  S  S  N  P  R  L  F  V  S  G  S
TGTGACACAACTGCTCGACTGTGGGACACCCGAGTTGCTAGTCGAGCTCAACGAACATTTCATGGTCATGAGGGAGATGTTAAT
 C  D  T  T  A  R  L  W  D  T  R  V  A  S  R  A  Q  R  T  F  H  G  H  E  G  D  V  N
ACTGTAAAGTTCTTCCCTGATGGTAATCGCTTTGGAACTGGTTCAGATGATGGAACCTGCAGATTATTTGACATCAGAACTGGA
 T  V  K  F  F  P  D  G  N  R  F  G  T  G  S  D  D  G  T  C  R  L  F  D  I  R  T  G
CACCAGCTGCAAGTATACTACCAGCCGCATGGTGACAGTGATATCCCTCATGTGACTTCCATGGCATTTTCTATCTCAGGCCGT
 H  Q  L  Q  V  Y  Y  Q  P  H  G  D  S  D  I  P  H  V  T  S  M  A  F  S  I  S  G  R
CTTCTCTTCGTCGGGTACTCTAATGCAGATTGTTACGTGTGGGACACCCTATTAGCAAAGGTGGTCCTAAACTTGGGAGCAGTT
 L  L  F  V  G  Y  S  N  A  D  C  Y  V  W  D  T  L  L  A  K  V  V  L  N  L  G  A  V
CAAAACTCTCATGAAGGGCGAATAAGTTGCCTGGGACTGTCAGCTGATGGAAGCGCCTTATGTACAGGAAGTTGGGATACTAAC
 Q  N  S  H  E  G  R  I  S  C  L  G  L  S  A  D  G  S  A  L  C  T  G  S  W  D  T  N
CTGAAGATTTGGGCTTTTGGTGGCACAGAAGTGtGA
 L  K  I  W  A  F  G  G  T  E  V  .
```

Figure 2. Complete coding sequences and amino acid sequence of Gβ2 in pepper. (*) = the stop codon.

The putative Gβ2 and ArcA1 proteins were also analyzed. Gβ2 conserved domains were identified as coiled-coil regions (positions 3 to 33) and WD40 domains (positions 54 to 93; 96 to 135; 145 to 185; 192 to 233; 237 to 276; 279 to 323; 330 to 369). ArcA1 conserved domains were identified as WD40 domains (positions 5 to 45; 53 to 92; 95 to 134; 136 to 180; 183 to 222; 225 to 262; 291 to 326).

YNPP1 Gβ2 and ArcA1 genes did not contain putative signal peptides (Bendtsen et

al., 2004; Deng et al., 2009). Transmembrane topology prediction revealed an internal N-terminus for YNPP1 Gβ2 with 2 strong transmembrane helices. ArcA1 also has an internal N-terminus with 1 strong transmembrane helix. For subcellular localization, analysis indicated 62.1% likelihood of Gβ2 microbodies and 64% likelihood of ArcA1 microbodies (both in the peroxisomes; Nakai and Horton, 1999).

```
ATGGCGCAAGAATCACTTGTCCTCCGCGGCACCATGAAAGCCCACACTGATTGGGTAACCGCCATTGCTACCCCAATTGAC
 M  A  Q  E  S  L  V  L  R  G  T  M  K  A  H  T  D  W  V  T  A  I  A  T  P  I  D
AACTCCGACATGATTGTCACTTCTTCGAGAGACAAATCCATCATTGTCTGGTCACTAACCAAAGACGGCCCAACATACGGT
 N  S  D  M  I  V  T  S  S  R  D  K  S  I  I  V  W  S  L  T  K  D  G  P  T  Y  G
GTCCCCCGCCGCCGTCTCACCGGACACGGCCACTTTGTCCAGGATGTCGTTCTTtCCTCCGACGGTATGTTCGCTCTTTCC
 V  P  R  R  R  L  T  G  H  G  H  F  V  Q  D  V  V  L  S  S  D  G  M  F  A  L  S
GGTTCATGGGATGGTGAGCTTCGTTTATGGGATCTTCAGGCGGGGACCACCGCTCGTAGGTTTGTTGGTCATACTAAGGAT
 G  S  W  D  G  E  L  R  L  W  D  L  Q  A  G  T  T  A  R  R  F  V  G  H  T  K  D
GTGCTATCCGTTGCGTTTTCGGTTGATAACCGTCAGATTGTTTCCGCGTCTAGGGACAAGTCGATTAAGCTGTGGAACACT
 V  L  S  V  A  F  S  V  D  N  R  Q  I  V  S  A  S  R  D  K  S  I  K  L  W  N  T
TTAGGTGAGTGTAAGTACACTATTCAGGATGGGGATTCGCATTCTGATTGGGTTTCGTGTGTGCGTTTTAGCCCGAATACG
 L  G  E  C  K  Y  T  I  Q  D  G  D  S  H  S  D  W  V  S  C  V  R  F  S  P  N  T
CTTCAGCCAACTATCGTTTCGGGTTCATGGGACCGTACTGTGAAGATATGGAACTTGACTAACTGTAAGCTGCGTTCGACT
 L  Q  P  T  I  V  S  G  S  W  D  R  T  V  K  I  W  N  L  T  N  C  K  L  R  S  T
CTTGCTGGACACACTGGGTATGTCAACACTGTGGCGGTTTCGCCTGATGGTTCGCTGTGTGCTAGTGGAGGGAAGGATGGA
 L  A  G  H  T  G  Y  V  N  T  V  A  V  S  P  D  G  S  L  C  A  S  G  G  K  D  G
GTTATTTTGCTGTGGGATTTGGCTGAGGGGAAGAAGCTGTACTCGCTTGACGCGGGTTCGATTATTCATACTCTTTGCTTT
 V  I  L  L  W  D  L  A  E  G  K  K  L  Y  S  L  D  A  G  S  I  I  H  T  L  C  F
AGTCCTAACAGGTACTGGCTTTGCGCCGCGACTGAGTCTAGCATTAAGATTTGGGATTTGGAGAGCAAGAGCATTGTGGTT
 S  P  N  R  Y  W  L  C  A  A  T  E  S  S  I  K  I  W  D  L  E  S  K  S  I  V  V
GATCTTCGAGTGGATCTCAAGCAAGAGAGTGAGATGCTTGCTGCCACTCCTGATGGAGGAGCTGCTCCTGTCAAGAACAAG
 D  L  R  V  D  L  K  Q  E  S  E  M  L  A  A  T  P  D  G  G  A  A  P  V  K  N  K
ATCATTTACTGTACCAGTTTGAGCTGGAGTGCTGATGGAAGCACACTTTTCAGTGGATATACAGATGGTTTGATTAGGGTC
 I  I  Y  C  T  S  L  S  W  S  A  D  G  S  T  L  F  S  G  Y  T  D  G  L  I  R  V
TGGGGTATTGGTCGTTATTAG
 W  G  I  G  R  Y  .
```

Figure 3. Complete coding sequences and amino acid sequence of ArcA1 in pepper. (*) = the stop codon.

Homology modeling

The 3-D structure of Gβ2 (1 to 292) was based on template 3iz6a (5.50 A) with 79.252% sequence identity; the 3-D structure of ArcA1 (1 to 247) was based on template 3dm0A (2.40 A) with 80.242% sequence identity (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006; Yu et al., 2010). The 3-D structure analysis may provide a basis for understanding the relationship between structure and function of Gβ2 and ArcA1.

Sequence analysis and evolutionary relationships

BLAST analysis revealed strong homology of Gβ2 with transcriptional regulators in 4 species (Figure 4); ArcA1 shares homology with transcriptional regulators in the species shown in Figure 5.

Phylogenetic trees were constructed based on the alignments as shown in Figures 6 and 7. YNPP1 Gβ2 is most closely related to Gβ2 of *Nicotiana tabacum* and ArcA1 is most closely related to its homolog in *N. plumbaginifolia*.

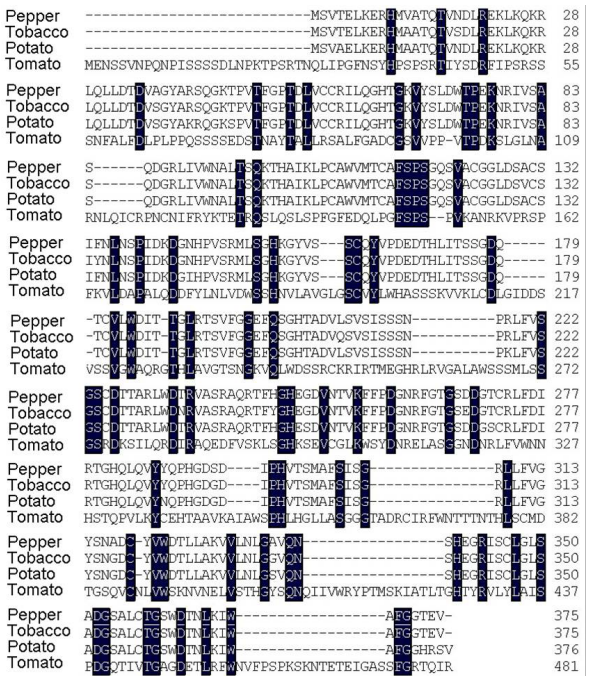

Figure 4. Multiple amino acid sequence alignment of Gβ2.

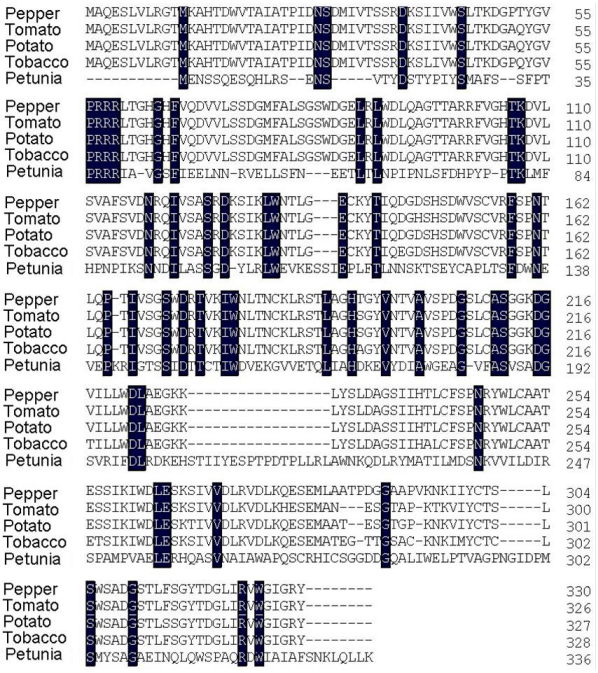

Figure 5. Multiple amino acid sequence alignment ArcA1.

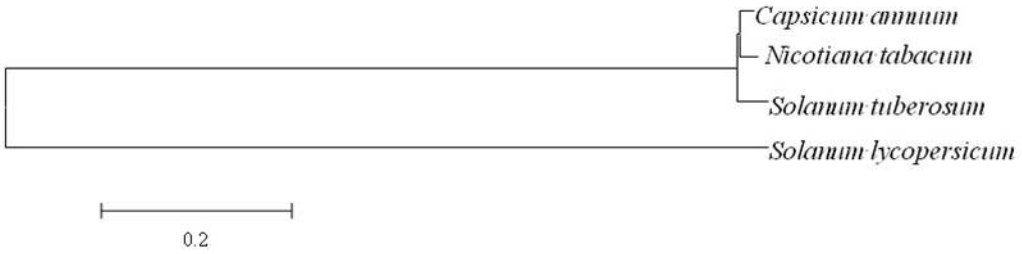

Figure 6. Phylogenetic tree of Gβ2 genes.

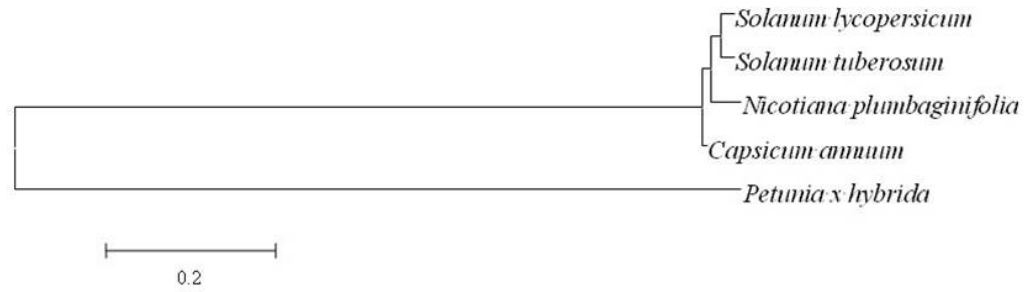

Figure 7. Phylogenetic tree of ArcA1 genes.

Tissue expression

The YNPP1 Gβ2 gene was highly expressed in the pericarp, moderately expressed in the stems, flowers, placenta, and leaves, and weakly expressed in seeds. There was no expression in the roots (Figure 8). The ArcA1 gene was moderately expressed in the pericarp and weakly expressed in seeds. There was no expression in the roots, stem, flowers, placenta, and leaves.

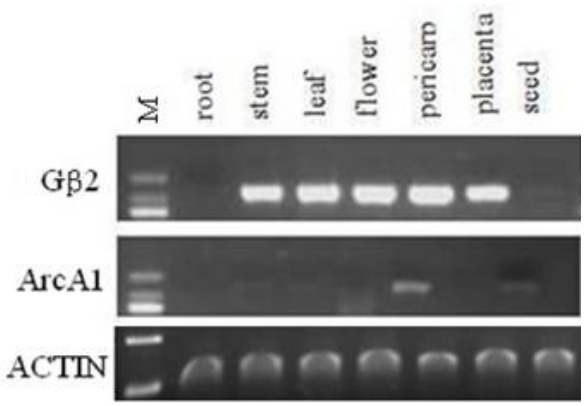

Figure 8. Tissue expression of pepper Gβ2 and ArcA1; actin served as the internal control.

DISCUSSION

Our analysis of the Gβ2 and ArcA1 amino acid sequences revealed 6 predicted WD repeats, which are remarkably well conserved among plants, yeast, nematodes, and humans, suggesting the functional importance of these domains. The WD-repeat unit is classified in a group of proteins composed almost entirely of WD repeat domains (Neer et al., 1994; Bassi et al., 1999). Proteins of this group are often seen in G protein β subunits (Fong et al., 1986) and other signal transduction-related proteins such as the receptor for activated protein kinase C (Liliental and Chang, 1998). Repeated WD40 motifs are a site for protein-protein interaction, and proteins containing WD40 repeats serve as platforms for the assembly of protein complexes or as mediators of transient interplay between proteins. Specificity is determined by sequences lying outside the repeats. Examples of such complexes are G proteins (beta subunit is a beta-propeller), TAFII transcription factor, and E3 ubiquitin ligase [(PubMed: 11814058), (PubMed: 10322433)]. In *Arabidopsis* spp, several WD40-containing proteins act as key regulators of plant-specific developmental events (Schultz et al., 1998; Letunic et al., 2012).

At the transcriptional level, the 2-component signal transduction system ArcAB modulates expression of many operons in response to the redox state of the environment (Lynch and Lin, 1996). ArcB is a transmembrane sensor kinase which, under anaerobic or microaerobic conditions, undergoes stable phosphorylation and transphosphorylates the response regulator ArcA (Kwon et al., 2000). The main targets for repression by the phosphorylated regulator are genes that encode enzymes involved in aerobic respiration, such as those of the tricarboxylic acid cycle. Cytochrome deoxidase, with its high affinity for oxygen, and fermentation enzymes such as pyruvate-formate lyase are activated under microaerobic conditions (Lynch and Lin, 1996).

Tissue expression analyses revealed higher expression of Gβ2 and ArcA1 in the pericarp, where anthocyanin was concentrated. Anthocyanin may correlate with Gβ2 and ArcA1 expression in response to tissue specificity and genetic background. Our experiment established the foundation for further research on these pepper genes.

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