**Differential isoform expression and protein localization from alternatively spliced *Apetala2*
in peanut under drought stress**

So-Yon Park* and Elizabeth Grabau

*Department of Plant Pathology, Physiology and Weed Science, 170 Drillfield Drive, Virginia Tech,
Blacksburg, VA, 24061, USA*

Key words: *Apetala2* (AP2), drought, peanut, alternative splicing

*Corresponding author (Email: soypark@vt.edu, Tel: 540-231-7132).

Summary

APETALA2 (AP2) belongs to the AP2/ Ethylene Responsive Factor (ERF) family and regulates expression levels of downstream stress responsive genes as a transcription factor. In this study, we cloned six different isoforms of *AhAP2* from peanut (*Arachis hypogaea*). Four isoforms (AhAP2.1, AhAP2.2, AhAP2.3 and AhAP2.4) had both AP2/ERF DNA binding domains and ERF-associated amphiphilic repression (EAR) motifs. Two isoforms (AhAP2.5 and AhAP2.6) only had an EAR suppressor domain. After agroinfiltration, AhAP2.1, AhAP2.3, and AhAP2.4 fused to yellow fluorescent protein (YFP) showed localization to the nucleolus, which is the site of transcription and ribosome biogenesis. AhAP2.2-YFP showed a dispersed signal in the nucleus. AhAP2.5 and AhAP2.6 fused to YFP localized to both the nucleus and cytoplasm. In addition, increased levels of *AhAP2.1* and *AhAP2.2* transcripts were observed in drought-treated peanut leaves, suggesting differential transcriptional regulation under drought stress conditions.

1. Introduction

Transcription factors (TFs) bind specific DNA sequences to control transcription and regulate responses under different conditions, including biotic and abiotic stress (Yamaguchi-Shinozaki and Shinozaki, 2006). APETALA2 (AP2) / Ethylene Responsive Factor (ERF) is one of the well-known families among ~2000 transcription factors in *Arabidopsis* (Mitsuda and Ohme-Takagi, 2009; Sakuma et al., 2002). AP2/ERF plays a role in regulation of primary and secondary metabolism in plant growth, development and responses to biotic/abiotic stress (Licausi et al., 2013). AP2/ERF is subdivided into the AP2 subfamily, dehydration-responsive element-binding protein (DREB) subfamily, ERF subfamily, related to ABI3/VP1 (RAV) subfamily and others (Sakuma et al., 2002).

In *Arabidopsis*, *AtAP2* (AT4G36920) has been reported as a regulator of floral development (Jofuku et al., 1994) and is composed of one or two AP2 domains (AP2-R1 and AP2-R2) (Kim et al., 2006; Licausi et al., 2013), ERF-amphiphilic repression (EAR) motifs (Ohta et al., 2001) and a microRNA 172 (miR172) binding site (Aukerman and Sakai, 2003). The EAR motif is a plant-specific repression domain (Licausi et al., 2013) that modulates transcription of target genes under plant stress and defense responses as a repressor (Hiratsu et al., 2003; Kazan, 2006; Ohta et al., 2001). The EAR motif is classified based on motif type (DLNxxP or LxLxL) and location (N-terminal, middle, or C-terminal region) (Kagale et al., 2010). The EAR motif is found in only 10 of 140 AP2/ERF genes (Licausi et al., 2013).

miR172 suppresses the transcript level of AP2 (Aukerman and Sakai, 2003); it regulates flowering and floral organ development through its action on the *AP2* transcription factor, which in turn activates and/or represses transcription of downstream genes (Chen, 2004). Recently, Wang et al. (2014) reported a new mechanism involving miR172 regulation of

soybean nodulation. Li et al. (2016) found that the transcript level of soybean *AP2* (Glyma01g39520 and target of miR172) was increased under drought stress, indicating that *AP2* is also involved in abiotic stress responses.

Alternative splicing plays a critical role in the post-transcriptional generation of multiple isoforms from a precursor, which leads to different protein variants (Filichkin et al., 2015). In previous reports, different phenotypes and patterns of *AP2* isoforms have been identified. For example, two mutants (splicing variants) of *AP2* showed phenotypes of semi-dwarf, shorter internodes, shorter, wider, wrinkled leaves, and smaller leaf angles than controls in maize (Jiang et al., 2012). *Arabidopsis* plants overexpressing water lily *AP2* showed significantly increased height compared to control plants (Luo et al., 2012). Two *Orchis italica* (Orchidoideae) *AP2* isoforms were differentially expressed in root, stem, leaf and inflorescence tissue (Salemme et al., 2013). Two different isoforms of kiwifruit *AP2* were reported (Varkonyi-Gasic et al., 2012). Transcript levels of barley, apple and maize *AP2* were detected from various tissues/organs including root and stem (Zhuang et al., 2011a; Zhuang et al., 2010; Zhuang et al., 2011b). These previous reports indicate that alternative splicing of *AP2* is a common phenomenon in plants.

In this study, we investigated peanut (*Arachis hypogaea*) *AhAP2* and cloned six different isoforms that differ in transcript structure due to alternative splicing. The isoforms all have different numbers of AP2/ERF DNA binding domains and EAR motifs. The six isoforms also showed different subcellular localization and transcript levels under drought stress.

2. Materials and methods

2.1 Plant material and water deficit conditions

Peanuts (*A. hypogaea*, Virginia type, commercial cultivar Phillips) were grown in a growth chamber under a regime of 16 hours light at 26°C /8 hours dark at 22°C. At twenty days of age, uniformly sized seedlings were selected for drought stress. To measure soil water content (%), all pots were weighed before and after withholding water up to 8 days. Three biological replicate leaves were collected at 0, 4 and 8 days after drought treatment.

2.2 RNA isolation and 5' and 3' RACE-PCR

Total mRNA was extracted from collected peanut leaves using TRIzol (Invitrogen, USA). Reverse transcription (RT) reactions followed manufacturer's instructions (New England Biolab, USA). Three technical replicates for each biological replicate were used.

5' and 3' rapid amplification of cDNA ends (RACE)-PCR were conducted using the GeneRacer™ kit (Invitrogen, USA) according to manufacturer's instructions. The first strand cDNA was synthesized by the SuperScript III reverse transcriptase (Invitrogen, USA). After 5' and 3' RACE, PCR products were gel-purified and cloned into pGEM T-easy vector (Promega, USA) for sequencing. Coding sequences of AhAP2 were aligned by Multiple Alignment using Fast Fourier Transform (<http://www.ebi.ac.uk/Tools/msa/mafft/>) (Fig. S1). All predicted amino acid sequences were aligned and compared by ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) (Fig. S2).

2.3 YFP plasmid constructs and agroinfiltration

The cDNAs of six *AhAP2* isoforms were amplified with gene specific primers (Supplemental Table 5), and cloned into pCR8/GW/TOPO (Invitrogen). The resulting clones

were recombined with pEarleygate104 (www.arabidopsis.org) using the LR Clonase II enzyme mix (Invitrogen, USA) and the T-DNA binary vectors were introduced into *A. tumefaciens* GV3101 strains. For agroinfiltration, *A. tumefaciens* GV3101 strains containing T-DNA binary vectors were infiltrated into intact 20-day old *N. benthamiana* leaves as previously described (Park et al., 2007). For localization studies, agroinfiltrated tissues were analyzed 3 days after infiltration using a Zeiss LSM 880 confocal microscope. For staining of plant cell nuclei, Hoechst 33342 (5 µg/mL) (Sigma, USA) was infiltrated into the leaves 20 min before confocal observation. Infiltration experiments were repeated at least three times.

2.4 Real-time quantitative PCR analysis

Real time qRT-PCR was performed in triplicate from independent biological replicates from stressed plants using SYBR mixture with ROX passive reference dye (Invitrogen, USA) and the StepOnePlus Real-Time PCR System (Invitrogen, USA). Dissociation curves were checked for specificity and quantification was analyzed by the $2^{-\Delta\Delta CT}$ method. Data were normalized through the expression levels of reference genes. *AhActin* and *18S rRNA* for qRT-PCR were used as internal controls (Table S1) (Dang and Chen, 2013; Mehta et al., 2013). The transcript levels at 0 days after drought treatment were set at 1 and data for other time point were presented as relative levels. All six isoforms were amplified by gene specific primers. All amplicons produced by qRT-PCR were sequenced and confirmed. Statistical analysis was carried out by using GraphPad Prism version 7.01 (GraphPad Software, USA). For significance tests, One-way ANOVA followed by Tukey's multiple comparison was used to reveal difference from 0 day control.

3. Results and discussion

3.1 Multiple isoforms of *AhAP2* transcription factor with AP2 domains and EAR motifs

Six different *AhAP2* isoforms were cloned from 5' and 3' RACE PCR. Each isoform showed distinct exon and intron structures as well as specific putative coding sequences and inferred polypeptide lengths (Fig. 1, Fig. S1 and Fig. S2). *AhAP2.1* transcript has 10 exons and 9 introns that are constitutively spliced. The *AhAP2.2* transcript lacks part of exon 9 due to an alternative 3' splice site in exon 9. *AhAP2.3* transcript retains intron 4. *AhAP2.4* transcript utilizes an alternative 3' splice site in exon 6, and retains introns 6 and 9. Both *AhAP2.5* and *AhAP2.6* transcripts result from use of an alternative 5' splice site in exon 1 and an alternative 3' splice site in exon 10 (Fig. 1). Using the 'Codewise' classifier program (Aghamirzaie et al., 2015), *AhAP2.5* and *AhAP2.6* were predicted as non-coding RNAs due to exon-skipping, however, these two isoforms also contained short open reading frames.

Inferred polypeptide lengths of *AhAP2.1* through *AhAP2.4* are 505, 365, 241, and 282 amino acids, respectively, if translated to functional polypeptides (Fig. S2). The sequence of *AhAP2.1* was similar to previous reports, exhibiting two AP2/ERF domains (AP2-R1 and AP2-R2) (Fig. S2, black lines) and two ethylene-responsive EAR motifs (Fig. S2, red box). *AhAP2.2* contains two AP2/ERF domains (AP2-R1 and AP2-R2) and two EAR motifs (DLNxxP and LxLxL type). *AhAP2.3* contains the first N-terminal EAR motif (DLNxxP) and an AP2-R1 domain. *AhAP2.4* has an N-terminal EAR motif (DLNxxP), a full AP2-R1 domain and an interrupted AP2-R2 domain.

Kagale et al. (2010) reported that most EAR motif sites were located in the C-terminal region and at slightly lower frequency in the N-terminal and middle regions. They also reported that the DLNxxP type motif was located in the C-terminal region more frequently than N-terminus, and that the LxLxL type motif was found in either N-terminus or C-terminus in *Arabidopsis*. In peanut, we found that two EAR motifs, DLNxxP type and LxLxL types, are located in the N-terminal and C-terminal regions in AhAP2, respectively. In addition, AhAP2 isoforms (AhAP2.3, AhAP2.4, AhAP2.5 and AhAP2.6) have only one AP2/ERF DNA binding domain or EAR motif. Thus, it appears that AhAP2 is regulated by alternative splicing and these isoforms may encode AP2 protein variants with or without EAR motifs and AP2/ERF domains. The function of the EAR motif has not been well studied in crop plants. Although further studies need to be conducted, we suggest that these structural variations would be excellent candidates for understanding the combinational function of AP2 domains and EAR motifs.

3.2 Visualization of AhAP2 isoforms fused to YFP in nucleoli, nucleus and cytoplasm

Previous studies reported that most AP2/ERF subcellular localization showed nuclear targeting (Andrianakaja et al., 2007; Krizek and Sulli, 2006; Lee et al., 2004; Luo et al., 2012). Mishra et al. (2013) have shown nucleolar localization of rice ERF proteins. In order to determine the subcellular localization of peanut AP2 isoforms, the AP2 cDNAs were fused with the gene for yellow fluorescent protein (YFP). The resulting plasmids were introduced into *Agrobacterium* and agroinfiltration assays were conducted into *N. benthamiana* to identify localization of YFP-AhAP2 isoforms. Confocal microscopic observation indicated that isoforms of AhAP2.1, AhAP2.3 and AhAP2.4 containing the AP2/ERF domain localized to the nucleolus (Fig. 2). Confocal Z-Stack images detected fluorescent signals of YFP-

AhAP2.1 in the nucleolus (red arrows, Fig. S3). Interestingly, another AP2/ERF domain-containing isoform, AhAP2.2, showed a dispersed signal in the nucleus (Fig. 2 and Fig. S4), different from AhAP2.1, AhAP2.3 and AhAP2.4.

AhAP2.5 and AhAP2.6 fused to YFP were located throughout in the cell, similar to localization of YFP alone (Fig. 3). Although these two isoforms were predicted as non-coding RNAs by computational analysis, they contain a putative EAR motif (DLNxxP type) (Fig. S2). Additional efforts are necessary to understand the different localization of six isoforms.

3.3 Gene expression of *AhAP2* isoforms

In soybean, an AP2 domain-containing gene (Glyma01g39520) showed an increase in transcript level under drought stress (Li et al., 2016). In order to determine whether the levels of AP2 isoforms are also affected by drought stress in peanut, transcript levels for specific isoforms were measured by qRT-PCR from leaves after 0, 4 and 8 days of drought treatment. Soil water content of day 4 and day 8 was 60% and 30%, respectively. The qRT-PCR analysis showed that at day 4, transcript levels of *AhAP2.1* and *AhAP2.2* were significantly increased (5.7 and 7.5 fold, respectively) while transcript levels of *AhAP2.3*, *AhAP2.4*, *AhAP2.5* and *AhAP2.6* were less than two fold elevated (Fig. 4). At the day 8, transcript levels of *AhAP2.1* and *AhAP2.2* were 5.3 and 2.4 fold increased compared to 0 day controls. Transcript levels of *AhAP2.3*, *AhAP2.4*, *AhAP2.5* and *AhAP2.6* were not significantly different at 8 days (Fig. 4). This result indicates that different splice variants of peanut AP2 are involved in drought stress response.

4. Conclusion

Fusion proteins from six isoforms of peanut AP2 with different numbers of AP2 domains and EAR suppressor motifs were localized in either nucleus, nucleolus or dispersed throughout the cell. In addition, we observed that two isoforms (*AhAP2.1* and *AhAP2.2*) were strongly induced under drought stress. qRT-PCR results from this study and previously reported soybean data (Li et al., 2016) support that *AP2* plays a role not only in floral organ development but also in abiotic stress in legume crops. Furthermore, we found that alternative splicing and different transcript levels of *AhAP2* are related to drought stress in *AhAP2*. This result reveals that an isoform-specific approach would be required to understand differential expression of *AP2* transcription factors. Different isoforms generate different polypeptides that may play different roles in regulation for target downstream genes. Further study of novel *AhAP2* isoforms should provide a better understanding of a transcription factor/downstream gene regulation through the combinations of AP2/ERF and EAR to improve crop growth and production under drought conditions.

Acknowledgements

This work was supported in part by the USDA National Institute of Food and Agriculture, Multistate research project S-1059. We thank Delasa Aghamirzaie for Codewise analysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

- Aghamirzaie, D., Batra, D., Heath, L.S., Schneider, A., Grene, R., Collakova, E., 2015. Transcriptome-wide functional characterization reveals novel relationships among differentially expressed transcripts in developing soybean embryos. *BMC Genomics* 16(1), 928.
- Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., de Carvalho-Niebel, F., 2007. AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. *Plant Cell* 19(9), 2866-2885.
- Aukerman, M.J., Sakai, H., 2003. Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *Plant Cell* 15(11), 2730-2741.
- Chen, X., 2004. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* 303(5666), 2022-2025.
- Dang, P., Chen, C., 2013. Modified method for combined DNA and RNA isolation from peanut and other oil seeds. *Mol. Biol. Rep.* 40(2), 1563-1568.
- Filichkin, S., Priest, H.D., Megraw, M., Mockler, T.C., 2015. Alternative splicing in plants: directing traffic at the crossroads of adaptation and environmental stress. *Curr. Opin. Plant Biol.* 24, 125-135.
- Hiratsu, K., Matsui, K., Koyama, T., Ohme-Takagi, M., 2003. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. *Plant J.* 34(5), 733-739.
- Jiang, F., Guo, M., Yang, F., Duncan, K., Jackson, D., Rafalski, A., Wang, S., Li, B., 2012. Mutations in an AP2 Transcription Factor-Like Gene Affect Internode Length and Leaf Shape in Maize. *PLoS ONE* 7(5), e37040.
- Jofuku, K.D., den Boer, B.G., Van Montagu, M., Okamoto, J.K., 1994. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell* 6(9), 1211-1225.
- Kagale, S., Links, M.G., Rozwadowski, K., 2010. Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. *Plant Physiol.* 152(3), 1109-1134.
- Kazan, K., 2006. Negative regulation of defence and stress genes by EAR-motif-containing repressors. *Trends Plant Sci.* 11(3), 109-112.
- Kim, S., Soltis, P.S., Wall, K., Soltis, D.E., 2006. Phylogeny and Domain Evolution in the APETALA2-like Gene Family. *Mol. Biol. Evol.* 23(1), 107-120.
- Krizek, B.A., Sulli, C., 2006. Mapping sequences required for nuclear localization and the transcriptional activation function of the Arabidopsis protein AINTEGUMENTA. *Planta* 224(3), 612-621.
- Lee, J.H., Hong, J.P., Oh, S.K., Lee, S., Choi, D., Kim, W.T., 2004. The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsicum annuum* L.) interacts in vitro with both GCC and DRE/CRT sequences with different binding affinities: possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants. *Plant Mol. Biol.* 55(1), 61-81.

- Li, W., Wang, T., Zhang, Y., Li, Y., 2016. Overexpression of soybean miR172c confers tolerance to water deficit and salt stress, but increases ABA sensitivity in transgenic *Arabidopsis thaliana*. *J. Exp. Bot.* 67(1), 175-194.
- Licausi, F., Ohme-Takagi, M., Perata, P., 2013. APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytol.* 199(3), 639-649.
- Luo, H., Chen, S., Jiang, J., Teng, N., Chen, Y., Chen, F., 2012. The AP2-like gene NsAP2 from water lily is involved in floral organogenesis and plant height. *J. Plant Physiol.* 169(10), 992-998.
- Mehta, R., Radhakrishnan, T., Kumar, A., Yadav, R., Dobaria, J.R., Thirumalaisamy, P.P., Jain, R.K., Chigurupati, P., 2013. Coat protein-mediated transgenic resistance of peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease through *Agrobacterium*-mediated genetic transformation. *Indian J. Virol.* 24(2), 205-213.
- Mishra, M., Kanwar, P., Singh, A., Pandey, A., Kapoor, S., Pandey, G.K., 2013. Plant omics: genome-wide analysis of ABA repressor1 (ABR1) related genes in rice during abiotic stress and development. *OMICS* 17(8), 439-450.
- Mitsuda, N., Ohme-Takagi, M., 2009. Functional Analysis of Transcription Factors in *Arabidopsis*. *Plant and Cell Physiology* 50(7), 1232-1248.
- Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., Ohme-Takagi, M., 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13(8), 1959-1968.
- Park, S.Y., Yu, J.W., Park, J.S., Li, J., Yoo, S.C., Lee, N.Y., Lee, S.K., Jeong, S.W., Seo, H.S., Koh, H.J., Jeon, J.S., Park, Y.I., Paek, N.C., 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. *Plant Cell* 19(5), 1649-1664.
- Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., Yamaguchi-Shinozaki, K., 2002. DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochem. Biophys. Res. Commun.* 290(3), 998-1009.
- Salemme, M., Sica, M., Iazzetti, G., Gaudio, L., Aceto, S., 2013. The AP2-like gene OitaAP2 is alternatively spliced and differentially expressed in inflorescence and vegetative tissues of the orchid *Orchis italica*. *PLoS ONE* 8(10), e77454.
- Varkonyi-Gasic, E., Lough, R.H., Moss, S.M.A., Wu, R., Hellens, R.P., 2012. Kiwifruit floral gene APETALA2 is alternatively spliced and accumulates in aberrant indeterminate flowers in the absence of miR172. *Plant Mol. Biol.* 78(4), 417-429.
- Wang, Y., Wang, L., Zou, Y., Chen, L., Cai, Z., Zhang, S., Zhao, F., Tian, Y., Jiang, Q., Ferguson, B.J., Gresshoff, P.M., Li, X., 2014. Soybean miR172c Targets the Repressive AP2 Transcription Factor NNC1 to Activate ENOD40 Expression and Regulate Nodule Initiation. *The Plant Cell* 26(12), 4782-4801.
- Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* 57(1), 781-803.
- Zhuang, J., Anyia, A., Vidmar, J., Xiong, A.-S., Zhang, J., 2011a. Discovery and expression assessment of the AP2-like genes in *Hordeum vulgare*. *Acta Physiologiae Plantarum* 33(5), 1639-1649.

Zhuang, J., Deng, D.-X., Yao, Q.-H., Zhang, J., Xiong, F., Chen, J.-M., Xiong, A.-S., 2010. Discovery, phylogeny and expression patterns of AP2-like genes in maize. *Plant Growth Regulation* 62(1), 51-58.

Zhuang, J., Yao, Q.-H., Xiong, A.-S., Zhang, J., 2011b. Isolation, Phylogeny and Expression Patterns of AP2-Like Genes in Apple (*Malus × domestica* Borkh). *Plant Molecular Biology Reporter* 29(1), 209-216.

Fig. 1. Predicted exon-intron organization of *AhAP2* isoforms. Blue boxes are predicted protein coding regions. Purple striped boxes are unspliced intron regions. Lines represent intron sequences.

Fig. 2. Localization of *AhAP2* isoforms in *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* GV3101 containing cDNAs for YFP-*AhAP2.1*, YFP-*AhAP2.2*, YFP-*AhAP2.3*, and YFP-*AhAP2.4* were infiltrated. YFP fluorescence is shown in the *first column*, bright field (BF) is shown in *second column*, Hoechst 33342 stained nucleic acids (DAPI) is shown in the *third column*, and the merged image is shown in the *fourth column*. Red bars indicate 5 μ m.

Fig. 3. Localization of *AhAP2.5* and *AhAP2.6* in *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* GV3101 containing cDNAs for YFP control, YFP- *AhAP2.5*, and YFP- *AhAP2.6* were infiltrated. YFP fluorescence is shown in the *first column*, bright field (BF) is shown in *second column*, Hoechst 33342 stained nucleic acids (DAPI) is shown in the *third column*, and the merged image is shown in the *fourth column*. Red bars indicate 20 μ m.

Fig. 4. qRT-PCR analysis of six *AhAP2* isoforms under drought stress. For all six isoforms, relative transcript levels were measured at 0, 4, 8 days of drought stress. *AhActin* and *18S rRNA* were used as internal controls. Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$). Error bars represent standard deviation. At least three biological

replicates for each sample and three technical replicates for each biological replicate were used for qRT-PCR analysis.

Fig. 1. Predicted exon-intron organization of *AhAP2* isoforms. Blue boxes are predicted protein coding regions. Purple striped boxes are unspliced intron regions. Lines represent intron sequences.

Fig. 2. Localization of AhAP2 isoforms in *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* GV3101 containing cDNAs for YFP-AhAP2.1, YFP-AhAP2.2, YFP-AhAP2.3, and YFP-AhAP2.4 were infiltrated. YFP fluorescence is shown in the *first column*, bright field (BF) is shown in *second column*, Hoechst 33342 stained nucleic acids (DAPI) is shown in the *third column*, and the merged image is shown in the *fourth column*. Red bars indicate 5 μ m.

Fig. 3. Localization of AhAP2.5 and AhAP2.6 in *Nicotiana benthamiana* leaves. *Agrobacterium tumefaciens* GV3101 containing cDNAs for YFP control, YFP- AhAP2.5, and YFP- AhAP2.6 were infiltrated. YFP fluorescence is shown in the *first column*, bright field (BF) is shown in *second column*, Hoechst 33342 stained nucleic acids (DAPI) is shown in the *third column*, and the merged image is shown in the *fourth column*. Red bars indicate 20 μ m.

Fig. 4. qRT-PCR analysis of six *AhAP2* isoforms under drought stress. For all six isoforms, relative transcript levels were measured at 0, 4, 8 days of drought stress. *AhActin* and *18S rRNA* were used as internal controls. Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$). Error bars represent standard deviation. At least three biological replicates for each sample and three technical replicates for each biological replicate were used for qRT-PCR analysis.