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Related to ABA-Insensitive3(ABI3)/Viviparous1 and AtABI5 transcription factor coexpression in cotton enhances drought stress adaptation

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Summary

Drought tolerance is an important trait being pursued by the agbiotech industry. Abscisic acid (ABA) is a stress hormone that mediates a multitude of processes in growth and development, water use efficiency (WUE) and gene expression during seed development and in response to environmental stresses. Arabidopsis B3-domain transcription factor Related to ABA-Insensitive3 (ABI3)/Viviparous1 (namely AtRAV2) and basic leucine zipper (bZIPs) AtABI5 or AtABF3 transactivated ABA-inducible promoter:GUS reporter expression in a maize mesophyll protoplast transient assay and showed synergies in reporter transactivation when coexpressed. Transgenic cotton (*Gossypium hirsutum*) expressing AtRAV1/2 and/or AtABI5 showed resistance to imposed drought stress under field and greenhouse conditions and exhibited improved photosynthesis and WUEs associated with absorption through larger root system and greater leaf area. We observed synergy for root biomass accumulation in the greenhouse, intrinsic WUE in the field and drought tolerance in stacked AtRAV and AtABI5 double-transgenic cotton. We assessed AtABI5 and AtRAV1/2 involvement in drought stress adaptations through reactive oxygen species scavenging and osmotic adjustment by marker gene expression in cotton. Deficit irrigation-grown AtRAV1/2 and AtABI5 transgenics had 'less-stressed' molecular and physiological phenotypes under drought, likely due to improved photoassimilation and root and shoot sink strengths and enhanced expression of endogenous GhRAV and genes for antioxidant and osmolyte biosynthesis. Overexpression of bZIP and RAV TFs could impact sustainable cotton agriculture and potentially other crops under limited irrigation conditions.

Introduction

Engineering drought tolerance is an objective of the agbiotech industry and has the potential to create novel drought insurance models (e.g. purposeful imposition of drought stress during vegetative growth). Plant responses to the environment are not comprised of linear signalling pathways, but rather a complex network evolved to deal with ever-changing environments and plants' immobility. Abscisic acid (ABA) is a stress hormone that mediates a multitude of processes in growth and development, water use efficiency (WUE) and gene expression during seed development and in response to environmental stresses. Seed maturation and freezing/drought/salt tolerance likely share common protective mechanisms, as they all involve dehydration stress. Genes encoding cold-responsive, salt-inducible and late embryogenesis-abundant protein homologues in wheat, maize, barley, carrot and the resurrection plant *Craterostigma* are induced by ABA and dehydration stress (Dure, 1993; Ingram and Bartels, 1996) and have also been named *RAB*s (Responsive to ABA), demonstrating the phenomenon of cross-tolerance to environmental stresses where exposure to one stress confers resistance to others. Although the exact roles of *RAB* genes in cross-tolerance have not yet been drawn, there are strong evidences that support their adaptive functions in desiccation, freezing and salt tolerance beyond the plant kingdom (Campos *et al.*, 2013; Thomashow, 1999). Altered expression of ABA signalling components can have utilitarian effects on stress adaptation of plants (Uno *et al.*, 2000).

Transcription factors (TFs) control virtually all plant traits including yield, disease resistance, cold and drought protection, and myriad value-added crop properties by coordinated regulation of multiple target genes of known or unknown functions. The Basic 3-DNA-binding domain (B3-DBD) TFs, first identified as the *viviparous1* (*vp1*) mutant from *Zea mays* (McCarty *et al.*, 1991; Suzuki *et al.*, 1997) and the orthologous Arabidopsis mutant *ABA-insensitive-3* (*abi3*) (Giraudat *et al.*, 1992), play key roles in a hierarchical cascade of TF interactions that control seed maturation (Finkelstein and Somerville, 1990; McCarty *et al.*, 1991) via ABA signalling. B3-DBD TFs have been classified into five gene families: *AUXIN RESPONSE FACTOR/ARF*, *ABI3*,

HIGH LEVEL EXPRESSION of SUGAR INDUCIBLE, *RELATED* to *ABI3/VP1* (*RAV*) and *REPRODUCTIVE MERISTEM* (Peng and Weselake, 2013; Romanel *et al.*, 2009). The DNA-binding specificity of B3-DBD TFs has been studied in ABI3, RAVs and ARFs, and the B3 domain of each family binds to a different target DNA sequence. The RAV/TEMPRANILLO family of TFs contains an N-terminal APETELA2 (AP2)-like DBD that binds 5′-CAACA-3′, whereas the B3-DBD binds 5′- CACCTG-3′ (Kagaya and Hattori, 2009). Various *RAVs* from several species are induced in response to multiple hormone treatments or stresses (Xu *et al.*, 2011), and overexpression of CaRAV1 in Arabidopsis or SlRAV2 in tomato results in the induction of pathogenesis-related genes, enhanced resistance against infection by bacterial pathogens and tolerance to osmotic, salt and cold stresses (Li *et al.*, 2011; Sohn *et al.*, 2006). Biotic and abiotic stresses generate reactive oxygen species (ROS) that destroy membrane lipids and promote cell death. Virus-induced or RNA interference gene silencing of *CaRAV1* resulted in higher levels of lipid peroxidation, supporting that CaRAV1 is involved in ROS scavenging (Lee *et al.*, 2010). Furthermore, CaRAV1 physically interacts with oxidoreductase1/CaOX1 in yeast two-hybrid experiments (Lee *et al.*, 2010).

There are 81 predicted basic leucine zipper (bZIP) TFs in Arabidopsis, but only one bZIP subfamily (ABA-INSENSITIVE-5/ABI5 and its close homologues ABA-responsive element-binding factors/ABF1-4) has been genetically or functionally linked to ABA response in a pathway from Pyrabactin-resistance-like/regulatory control of ABA receptors (PYR/RCAR) and downstream SnRK2-like protein kinases and type 2C protein phosphatases (Cutler *et al.*, 2010; Lynch *et al.*, 2012; Okamoto *et al.*, 2013; Raghavendra *et al.*, 2010; Soon *et al.*, 2012). ABI5 is involved in seed-specific responses, whereas the ABFs play roles at the seedling and later stages. ABI5/ABFs are subject to proteolytic regulation by ubiquitylation mediated by 14-3-3 proteins and multiple E3 ligases (Chen *et al.*, 2013). At the seedling stage, ectopic expression of ABI5 leads to higher expression of stress-induced genes (e.g. *Cor6.6*, *Cor15a*, *Rab18*) and overexpression is sufficient to confer hypersensitivity to exogenous ABA, which inhibits root growth (Brocard *et al.*, 2002; Lopez-Molina *et al.*, 2001). ABI5 overexpression also results in high sensitivity to glucose and anthocyanin accumulation in response to sugar stress (Finkelstein *et al.*, 2002). Analyses of transcript accumulation in *abi5* mutants suggest that, similar to ABI3, ABI5 has both activator and repressor functions that may have either synergistic or antagonistic effects on gene expression, depending on the target gene. ABI5 protein accumulation is further enhanced by ABA-induced phosphorylation and resulting stabilization of the protein (Lopez-Molina *et al.*, 2001; Wang *et al.*, 2013).

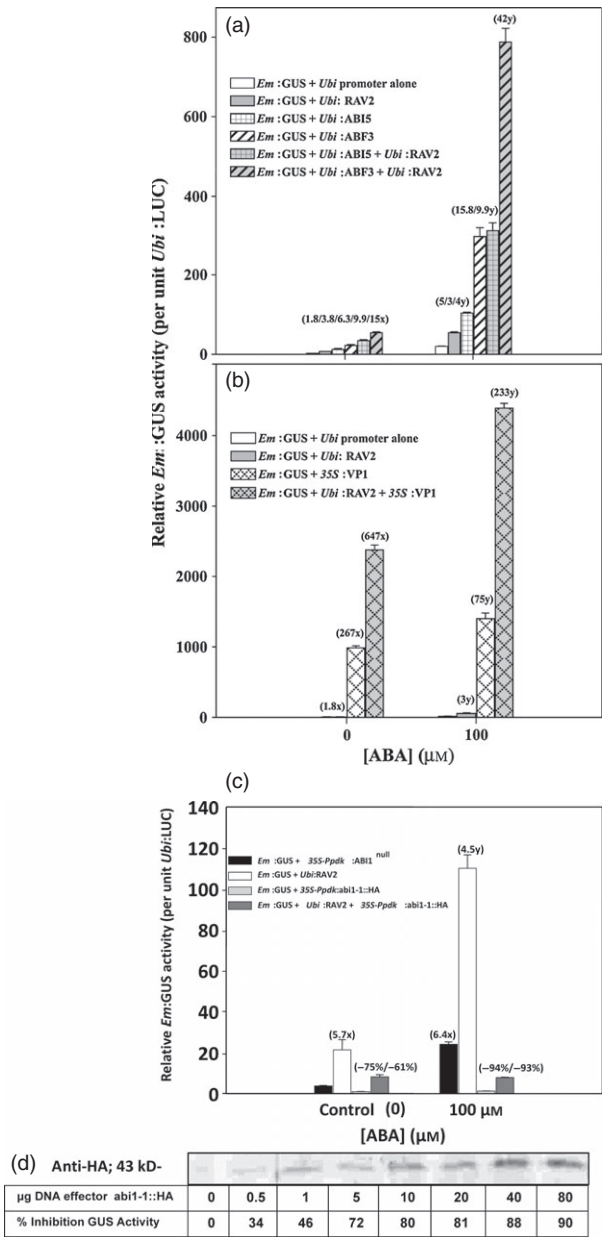
In the present study, we show that B3-DBD effector RAVs, and ABA effector bZIPs transactivate ABA-inducible gene expression in a maize mesophyll protoplast transient assay and show synergy in their activities when coexpressed. Importantly, transgenic cotton expressing AtRAV1 or AtRAV2 and/or ABI5 TFs showed resistance to imposed drought stress under greenhouse and field conditions and had improved photosynthetic efficiency, likely due to improved absorption through a larger root system. Transgenic cotton expressing Arabidopsis RAVs and ABI5 has a ‘less-stressed phenotype’, which may have broad utility for engineering abiotic stress tolerance in crops.

Results

Functional interactions of AtRAV2 with ABA effectors in transiently transformed maize mesophyll protoplasts

We previously showed by cotransformation of reporter and multiple effector plasmid constructs (all components in *trans*) that bZIP TFs ABI5 and ABF3 interact functionally and physically with maize B3-DBD TF VP1 (Finkelstein *et al.*, 2005), and that a homozygous T-DNA insertion line (SALK_070847) in B3-DBD TF *AtRAV2/At1g68840* shows elevated transpiration rates (Luo, Gampala, and Rock. 2005. 16th Intl. Conf. Arabidopsis Res. http://www.arabidopsis.org), suggesting the involvement of RAVs in ABA responses. Similarly, an AtRAV1/At1g13260 homozygous T-DNA insertion line (SALK_021865) showed modest ABA insensitivity to root growth inhibition, and conversely, overexpression of AtRAV1 or AtRAV2 in transgenic Arabidopsis resulted in increased sensitivity to ABA inhibition of seed germination and root growth (data not shown; Mittal, 2012). To further test the involvement of AtRAV2 as a positive effector of ABA signalling, we overexpressed the AtRAV2 cDNA in transiently transformed maize mesophyll protoplasts (Jia *et al.*, 2009; Sheen, 2001) and characterized its function on ABA-inducible reporter gene expression and interactions with known positive effectors (ABI5, ABF3, VP1) and the dominant-negative ABA effector mutant abi1-1 encoding a protein phosphatase type 2C (PP2C) (Sheen, 1998). The results are shown in Figure 1. The ABA effectors ABI5 and ABF3 (Finkelstein *et al.*, 2005) resulted in transactivation of between four- and 16-fold above promoter of *Early-methionine-rich*:GUS reporter gene (*Em*:GUS) alone in the absence or presence of a saturating concentration (100 μM) ABA, respectively. ABA treatment resulted in fivefold induction of the *Em*:GUS reporter alone, whereas overexpression of AtRAV2 resulted in transactivation of two- to threefold above the reporter gene alone in both the presence and absence of exogenous ABA (Figure 1a), values somewhat less than overexpression of ABI5 or ABF3. Similar to previous results with B3-DBD TF VP1 and bZIPs (Finkelstein *et al.*, 2005), we observed a synergy between AtRAV2 and ABI5 or ABF3 when cotransformed, where the observed transactivation was 10-fold and 42-fold for RAV2 plus ABI5 and RAV2 plus ABF3, respectively, above reporter gene alone in the presence of ABA. The interaction can be described as synergistic because the observed effects were about twice the sums of the two respective individual transgene effects in both the absence and presence of ABA (Figure 1a). Unexpectedly, we also observed a synergistic interaction between overexpressed B3-DBDs AtRAV2 and VP1 (Figure 1b).

To further characterize the functional interaction between AtRAV2 and known ABA signalling components, we tested whether the upstream PP2C dominant-negative mutant protein abi1-1, which abolishes phosphatase activity and physically interacts with Pyrabactin-like ABA receptors (Fujii *et al.*, 2009), SnRK2, CPK11, and possibly bZIP TFs (Antoni *et al.*, 2012; Lynch *et al.*, 2012), could specifically antagonize the AtRAV2 transactivation of the ABA-inducible *Em*:GUS reporter. Figure 1(c) shows the result that cotransformation of *abi1-1* effector with *AtRAV2* construct strongly antagonized the >fourfold-specific RAV2 transactivation of ABA-inducible reporter gene expression in the presence and absence of ABA (>90% inhibition for ABA treatment). Figure 1(d) shows an immunoblot of the dose dependence of input DNA on protein expression of abi1-1:: HA-tagged effector and excellent concordance of abi1-1 protein



μg DNA effector abi1-1::HA	0	0.5	1	5	10	20	40	80
% Inhibition GUS Activity	0	34	46	72	80	81	88	90

Figure 1 Synergistic interactions between known effectors of ABA signalling and AtRAV2 in transiently transformed maize mesophyll protoplasts. Numbers in parentheses (*x*) represent fold induction over 'No ABA' control, and numbers in parentheses (*y*) represent fold transactivation by over-expressed AtRAV2 (and/or other effector[s]) compared to treatment with 100 μM ABA alone. (a) Synergy between B3 domain-containing AtRAV2 and bZIPs AtABI5 and AtABF3. (b) Synergy between B3-domain ZmVP1 and AtRAV2. (c) Antagonism of AtRAV2 transactivation by upstream ABA effector abi1-1 dominant-negative (G180D) protein phosphatase 2C mutant. Negative numbers in parentheses indicate the per cent inhibition of pro*Em*:GUS expression relative to controls (without/with RAV2 cotransformation, respectively) by cotransformation of *abi1-1* construct. (d) Dose dependence of input effector DNA for abi1-1 antagonism of ABA (and AtRAV2) transactivation, shown by immunoblot of abi1-1::HA-tagged effector and corresponding per cent decreases in relative GUS activities measured in the same protoplast extracts. Error bars are ± SEM (n = 4).

expression to inhibition of *Em*:GUS reporter gene expression. Taken together, these results suggest that AtRAV2 overexpression can positively impact ABA responses in monocot and dicot plants, so we generated transgenic cotton overexpressing AtABI5 and AtRAVs to test the effects on drought stress tolerance.

Generation and molecular characterization of transgenic cotton lines expressing AtRAV1, AtRAV2, AtRAV2L and AtABI5

The pro*35S*-:RAV1, pro*35S*-:RAV2, pro*35S*-:RAV2L and pro*35S*-:ABI5 constructs were introduced into cotton (*Gossypium hirsutum*) cultivar Coker312, which has a high capacity for regeneration from hypocotyl explants, via Agrobacterium-mediated transformation (Bayley *et al.*, 1992). A total of four independent RAV1, eight RAV2, three RAV2L and two ABI5 transgenic events were propagated in the greenhouse and subjected to RNA blot analysis to confirm stable expression (Figure S1). Several larger transcripts than the sizes of full-length coding sequences (RAV2 = 1059 nt; RAV2L = 1086 nt; RAV1 = 1035 nt; ABI5 = 1329 nt) were consistently detected in independent transgenic lines, similar to our prior results in protoplasts (Jia *et al.*, 2009) where we observed transcription termination at the bovine growth hormone genomic polyadenylation signal (Goodwin and Rottman, 1992), but also predicted some read-through past this animal *cis* element based on bioinformatic analyses (Loke *et al.*, 2005). Homozygous transgenic plants from these lines and one kanamycin-selected noneffector transgenic (KSNT, a regenerant line from RAV2L transformation experiments that either subsequently lost the effector DNA or was a false positive for Kan^r) were subjected to semi-quantitative reverse transcriptase PCR (RT-PCR) (Figure 2) in the T_4 generation to validate the effector transgene expression over several generations and to identify high expression lines for physiological assays.

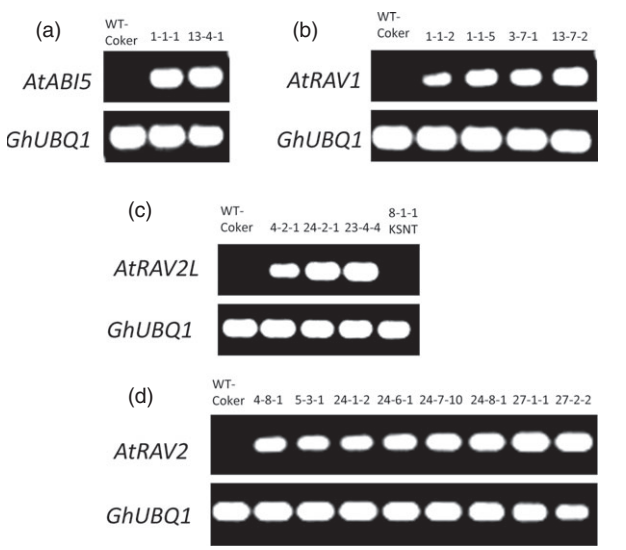


Figure 2 Transcript expression analysis for transgenes in cotton lines by reverse transcriptase-PCR. *GhUBQ1* was used as an internal control. (a) *AtABI5* over-expression in two independent lines compared with wild type. (b) *AtRAV1* over-expression in four independent lines compared with wild type. (c) *AtRAV2L* over-expression in three independent lines compared with wild type. (d) *AtRAV2* over-expression in eight independent lines compared with wild type.

Physiological characterization of cotton transgenic lines

All of the RAV2L and ABI5 overexpressing lines, and all but one of the RAV2 lines (24-7-10), generated higher seed cotton yield (fibre plus seed weight per plant) under WW greenhouse conditions (Figure S2A), whereas RAV1 and KSNT control regenerant line produced on par with wild-type Coker312. When tested in the field under the most extreme drought and heat conditions on record (2011) that resulted in a 43% overall yield reduction, deficit-irrigated (DI) transgenics, to a greater extent than WW transgenics (including RAV2$^{24\text{-}7\text{-}10}$), had yields on par with control genotypes (Figure S2B). Differences in yields for transgenics between greenhouse and field may have been due to the effects of RAV and ABI5 transgenes on phenology (Castillejo and Pelaz, 2008; Wang *et al.*, 2013); the phenotypes relating to flowering and fibre development will be described elsewhere. Leaf area was higher in transgenic lines under both WW and drought stress treatments in the field (Table 1). Both AtRAV1 cotton lines showed significant ($P < 0.04$) increases in leaf area per plant with concomitant significant increases in dry mass. Similar results were obtained with AtABI5 and AtRAV2/2L overexpressing lines (Table 1). These results showing increased leaf biomass and nonsignificant yield penalties during drought in the field suggested that RAV and ABI5 overexpressing lines may have higher WUE, possibly due to enhanced ABA response consistent with the protoplast transient expression results. Therefore, we analysed photosynthetic assimilation rates (***A***) in transgenics in response to water deficit under greenhouse and field conditions.

RAV1, RAV2 and ABI5 transgenic cotton maintains higher photosynthesis resulting in increased WUE, and RAVxABI5 stacked lines adapt better to drought treatments than individual parents

We analysed drought resistance in the transgenics by conducting controlled DI experiments in both greenhouse and the field. ***A*** and other parameters (stomatal conductance G_s, transpiration) were measured on control and transgenic event lines RAV1$^{13\text{-}7\text{-}2}$, RAV2$^{24\text{-}8\text{-}1}$, ABI5$^{13\text{-}4\text{-}1}$ and F_1 plants from RAVxABI5 crosses (RAV1$^{13\text{-}7\text{-}2}$xABI5$^{13\text{-}4\text{-}1}$ and RAV2$^{4\text{-}8\text{-}1}$xABI5$^{13\text{-}4\text{-}1}$) over a span of drought progression and recovery by re-watering in the greenhouse. The results for ***A*** are shown in Figure 3(a). Control Coker312 plants had a strong wilting phenotype in the afternoon on days 9, 10 and 11 of withholding water. The strong drought stress resulted in significant inhibitory effects on ***A*** compared to 7-day drought, when no signs of afternoon wilting were observed either in wild-type or transgenic plants. All the transgenic lines showed significantly better ***A*** under drought stress, especially at 11 days of drought (except ABI5$^{13\text{-}4\text{-}1}$ which also showed higher ***A,*** but at $P = 0.14$; Data S1), and did not show severe wilting symptoms upon withholding water. The stacked double-transgenic RAV1xABI5 cross showed the best ***A*** at 9, 10 and 11 days of drought and importantly outperformed its parental lines. RAV2xABI5 F_1 resulted in better ***A*** at 9- and 10-day drought and outperformed its parental lines at these time points. Re-watering resulted in all the transgenics having significantly higher ***A*** recovery after 16 h as compared to wild type. Importantly, RAV1xABI5 showed better ***A*** recovery over its parental lines. All the transgenic lines and wild type recovered near full photosynthetic capacity 5 days after re-watering. Interestingly, wild-type plants never reached the same level of photosynthesis as transgenics (Figure 3a) alluding to higher WUE in transgenics as predicted. Figure S3 shows the wilty phenotype seen in control Coker312 plants in the evening after 10-day drought treatment compared to individuals of RAV1xABI5 stacked double-transgenic line, which recovered by evening from afternoon wilting.

To further substantiate the transgene effects on photosynthesis under drought stress, we measured parameters in two independent field trials (Data S1). Figure 3(b, c) shows reproducible, significantly higher intrinsic WUE (carbon fixed per unit water transpired, G_s) over two field trials for nearly all transgenics under drought stress (Figure 3b), as well as synergistic effects of stacked RAVxABI5 double-transgenics under water-sufficient but extreme heat conditions (Figure 3c) and under DI (Data S1). To further characterize the physiological consequences of improved WUE, we quantified shoot and root biomass in transgenics under simulated repetitive DI conditions in the greenhouse.

Table 1 Leaf area (cm^2/plant) of transgenic lines grown 85 days in the field under well watered or deficit irrigation after day 42

	Well Watered			Deficit Irrigation		
Genotype	Average	%ΔWT†	±SEM*	Average	%ΔWT†	±SEM*
Coker312	2204		223	1611		115
ABI5$^{1\text{-}1\text{-}1}$	3626	64.5	714	**2596**‡	61.1	262
ABI5$^{13\text{-}4\text{-}1}$	2494	13.2	87	1945	20.8	252
RAV1$^{1\text{-}1\text{-}5}$	3559	61.5	507	**3002**‡	86.3	319
RAV1$^{13\text{-}7\text{-}2}$	**4108**§	86.4	99	**2805**‡	74.1	274
RAV2$^{4\text{-}8\text{-}1}$	3092	40.3	412	**2181**‡	35.4	172
RAV2$^{5\text{-}3\text{-}1}$	3231	46.6	602	2051	27.3	468
RAV2$^{24\text{-}8\text{-}1}$	**5103**‡	131.5	519	2606	61.7	399
RAV2L$^{4\text{-}2\text{-}1}$	**3103**‡	40.8	184	1778	10.4	282
RAV2L$^{24\text{-}2\text{-}1}$	3001	36.2	744	**2211**‡	37.2	159

Values in bold are significantly different from control Coker312.

*Standard error of the mean. $n = 3$ except for Coker312: $n = 12$.

†Average per cent change of transgenic line from wild-type Coker312.

‡Significantly different than Coker312 control, $P < 0.05$.

§Significantly different than Coker312 control, $P < 0.0001$ (two-tailed Student's *t*-test).

AtABI5, AtRAVs and ABI5xRAVs stacked double-transgenic cotton plants have longer internodes, accumulate higher total dry biomass, and especially roots, compared to wild type under deficit irrigation treatments

Cotton plants subjected to drought stress in the greenhouse resulted in reduced internode lengths manifest as reduced height (Figure S4). All the transgenic lines subjected to 90 days of repeated DI grew taller than wild type due to increased internode length (Table S1). Both ABI5 lines showed significant increases (6% and 18%) in internode length, whereas four of seven RAV2 lines showed 12%–21% significant increases ($P < 0.03$) and two others near significant increases ($P = 0.08$). All the RAV2 lines had correspondingly higher stem weight (Table S2). Figure S5 shows a representative transgenic RAV2 line with 17% increased internode length compared to wild-type Coker312. RAV1 lines had 5.8%–7.9% increases in internode length and (Table S1) significant increases in stem weight (9.7%–33%; Table S2).

WW control Coker312 (WW Coker) generated 52% more biomass ($P < 2 \times 10^{-7}$) compared with DI Coker312, which clearly demonstrated the efficacy of the DI treatments (Figure S4). ABI5$^{13\text{-}4\text{-}1}$ and the two ABI5$^{13\text{-}4\text{-}1}$xRAV1 double-transgenic lines

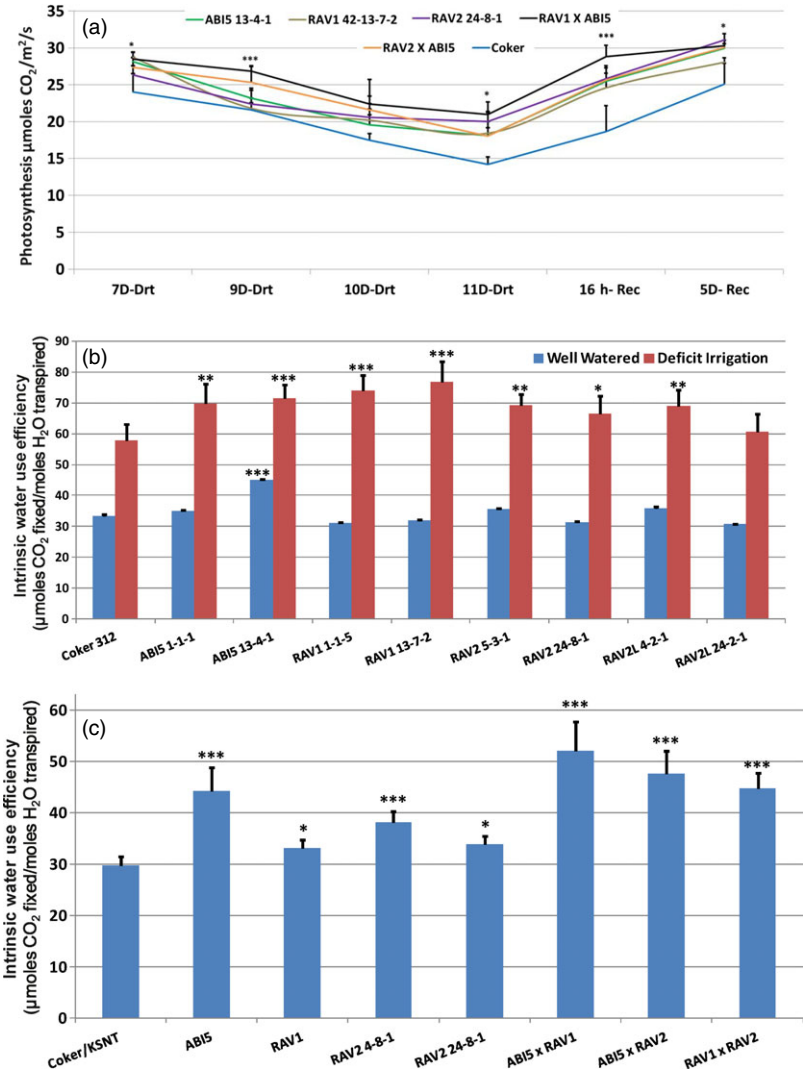


Figure 3 Photosynthetic parameters in stacked and single transgene cotton under drought stress. (a) Elevated ***A*** of transgenics grown in greenhouse, relative to control Coker312 during several days imposed drought stress and recovery. (b) Significantly higher intrinsic WUE in single gene transgenics in the field (2011) under WW (blue bars) and DI conditions (red bars). (c) Significantly higher intrinsic WUE in single gene and stacked double-gene transgenics in the field (2013) at peak bloom under conditions of marginal heat- and water-limiting stress. Error bars ± are SEM. (n = 5 [a, c]; n = 9 [b]). *Significantly different from control Coker312/KSNT at $P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ (two-tailed Student's t-test, equal variance assumed).

generated approximately 17%, 11% and 16% significantly higher total dry biomass, respectively (Table S3). Importantly, the quasi-control KSNT line showed an 8% significant reduction in total dry mass compared with wild type, supporting that the transgenic effects observed are effector mediated and not associated with somaclonal variation possible during the regeneration of plants from hypocotyl explants. We next analysed root biomass to investigate the basis of observed WUE increases.

Well-watered (WW) Coker312 generated a mere 5% greater root mass compared with DI Coker312, and the increase was nonsignificant (P = 0.66). Compared to the above-described 52% significant elevation in total dry mass production for full water vs. deficit (Figure S4), the marginal effect of DI on root biomass shows that the sink strength of roots is very strong under drought stress, an adaptation advantageous for cotton (Pace *et al.*, 1999). Both ABI5 lines, two of three RAV1 lines, six of seven RAV2 lines, two of three RAV2L lines and all RAVxABI5 stacked double-transgenic lines grew 21%–96% more root mass under DI compared with wild type (Table 2). Figure S6 and Table 2 show several cases of higher root mass phenotypes for multiple independent RAV1 and RAV2 transgenic cotton lines and the compelling synergistic effects of ABI5$^{13\text{-}4\text{-}1}$xRAV2$^{4\text{-}8\text{-}1}$ stacked double-transgenes compared to individual single transgene parents. As an independent validation of the results, the quasi-control KSNT line showed a 25% significant reduction ($P < 0.05$) in root biomass accumulation under DI (Table 2), further substantiating the transgene-specific effects on root growth.

Characterization of molecular marker expression associated with drought resistance in RAV and ABI5 transgenic lines

ABI5 overexpressing Arabidopsis plants, in response to ABA, accumulate transcripts of stress-inducible genes (e.g. *Cor78*, *Cor6.6*, *Cor15a* and *Rab18*) (Brocard *et al.*, 2002) and show high sensitivity to sugar stress resulting in anthocyanin accumulation (Finkelstein *et al.*, 2002), suggesting ABI5 overexpression promotes vegetative stress adaptation. To further test the hypothesis that enhanced stress adaptation is due to elevated ABA response, we quantified the expression of cotton At*RAB18* homologue *GhRAB18*. In Arabidopsis, *RAB18* accumulates in response to drought and cold stress in an ABA-dependent manner (Lång and Palva, 1992; Mantyla *et al.*, 1995). A *GhRAB18* transcript of low abundance was detected under WW conditions in cotton (Figure 4). *GhRAB18* transcript was induced approximately two-fold in response to drought (Figure 4; compare lanes 6 and 12). Re-watering did not alter the transcript level compared to

Table 2 Root* dry mass (g) in transgenic cotton lines subjected to 90 days of deficit irrigation in greenhouse conditions

Genotypes	Average	%Δ WT	SEM (+/−)	*P*-value
ABI5$^{1\text{-}1\text{-}1}$	6.6	38.6	0.6	0.007
ABI5$^{13\text{-}4\text{-}1}$	6.3	**31.7**	0.8	0.036
RAV1$^{1\text{-}1\text{-}5}$	8.7	81.9	0.8	0.000006
RAV1$^{3\text{-}7\text{-}1}$	6.2	30.5	0.4	0.039
RAV1$^{13\text{-}7\text{-}2}$	5.3	11.6	0.5	0.372
RAV2$^{4\text{-}8\text{-}1}$	5.8	**21.4**	0.4	0.107
RAV2$^{5\text{-}3\text{-}1}$	4.7	**−1.0**	0.6	0.9
RAV2$^{24\text{-}1\text{-}2}$	5.8	22.5	0.4	0.061
RAV2$^{24\text{-}6\text{-}1}$	6.3	31.7	0.4	0.011
RAV2$^{24\text{-}7\text{-}10}$	9.3	96.1	0.4	0.0000002
RAV2$^{24\text{-}8\text{-}1}$	8.4	77.1	1.3	0.00044
RAV2$^{27\text{-}2\text{-}2}$	7.1	49.7	0.9	0.003
RAV2L$^{4\text{-}2\text{-}1}$	6.3	32.7	0.5	0.020
RAV2L$^{23\text{-}4\text{-}4}$	6.0	25.0	0.8	0.084
RAV2L$^{24\text{-}2\text{-}1}$	4.5	−6.2	0.1	0.6
RAV1$^{1\text{-}1\text{-}5}$xABI5$^{13\text{-}4\text{-}1}$	7.5	58.3	0.9	0.001
RAV1$^{13\text{-}7\text{-}2}$xABI5$^{13\text{-}4\text{-}1}$	6.5	36.3	0.7	0.026
RAV2$^{4\text{-}8\text{-}1}$xABI5$^{13\text{-}4\text{-}1}$	7.2	**50.2**	0.7	0.001
RAV2$^{5\text{-}3\text{-}1}$xABI5$^{13\text{-}4\text{-}1}$	7.1	**48.1**	1.7	0.045
Wild-type Coker312	4.8		0.5	
KSNT	3.6	−25.1	0.4	0.042

*Root mass refers to lateral root mass excluding taproot.

Values in bold are shown for parental lines (RAV2 and ABI5).

The underlined bold values are shown for crossed progeny (RAV2xABI5).

For SEM, $n = 6$. For statistical significance analysis, a two-tailed Student's *t*-test was applied and nonequal variance assumed.

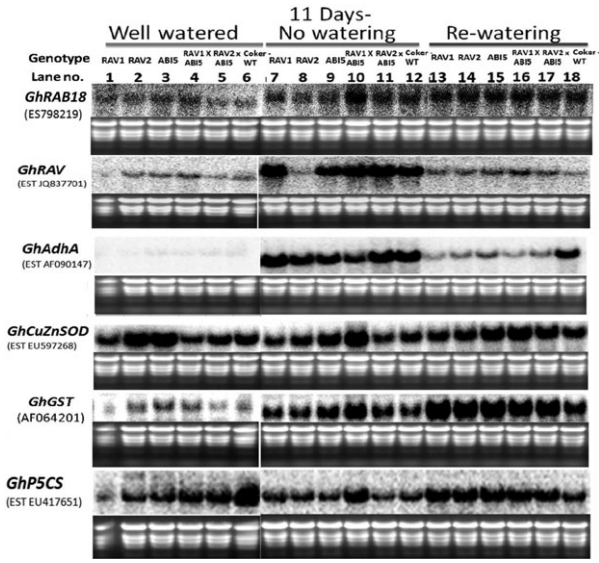


Figure 4 RNA blot assay for molecular marker gene expression associated with stress resistance/adaptation in RAV1$^{13\text{-}7\text{-}2}$-, RAV2$^{24\text{-}8\text{-}1}$- and ABI5$^{13\text{-}4\text{-}1}$-overexpressing and stacked double-transgenic lines. EtBr-stained gel of samples show equal loading. Lanes 1–6, 7–12 and 13–18 represent WW (24 DAS), 11 days of no watering (35 DAS) and overnight recovery (after re-watering) conditions, respectively.

drought (lanes 12 and 18), suggesting a role for *GhRAB18* in stress adaptation and recovery in cotton. Remarkably, the expression of *RAB18* was higher in RAV1 (approximately 1.3-fold), ABI5 (approximately 1.5-fold) and RAV1xABI5 (approximately 1.5-fold) transgenics under WW conditions. Importantly, expression was approximately 1.6-fold higher in RAV1xABI5 compared to Coker312 in response to drought, supporting that stress adaptation in RAV1xABI5 is associated with an elevated ABA response.

To further explore the molecular basis of improved performance of transgenic cotton lines under DI and test the hypothesis that the transgenic lines have a 'less-stressed' or stress-adapted phenotype, we examined transcript abundances of several stress-related marker genes. The endogenous *GhRAV* transcript was detected at low abundance under WW conditions and was highly induced by drought treatment. After 11 days of severe drought, *GhRAV* transcript expression was induced by approximately sixfold in wild-type Coker312 compared to WW conditions (Figure 4). *GhRAV* transcript abundance was reduced approximately 90% in response to re-watering (compare lane 18 with lane 12). Interestingly, endogenous *GhRAV* transcript was approximately 1.9–2.7 times more highly expressed in all the single transgenics and stacked double-transgenics in the recovery stage (Figure 4, compare lanes 13–17 to lane 18). This result supports that endogenous GhRAV plays a role in drought response and recovery, possibly by functional interactions with AtRAV and AtABI5.

With the onset of anoxia and biotic or abiotic stress, a rise in cytosolic Ca^{2+} acts as a signal for up-regulation of alcohol dehydrogenase (*Adh*) transcript in maize cells (Taiz and Zeiger, 2010). Figure 4 shows that the *GhAdhA* transcript is of low abundance under WW conditions (lanes 1-6), and was highly induced in response to drought (approximately 40-fold; lane 12 compared to lane 6). Furthermore, expression was reduced to half in response to re-watering (lane 18 compared to lane 12). Remarkably, all the transgenic and double-transgenic lines showed lower expression of *GhAdhA* in recovery stage (30–60% lower; lanes 13–17 compared to lane 18). Lower *GhAdhA* levels in transgenics under different treatments corroborate the notion of a 'less-stressed phenotype' and correlate with higher photosynthesis and WUE under drought stress and recovery (Figure 3, Data S1). In every case (transgenic lines and treatment) where endogenous transcript levels of *GhRAV* were high, the transcript levels of *GhAdhA* were lower (Figure 4), supporting a role for GhRAV in reducing stress.

Biotic and abiotic stresses generate excess ROS, which oxidize membrane lipids. ROS scavenging in plants involves superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (Apel and Hirt, 2004). There are numerous reports that show enhanced CuZnSOD and APX levels result in stress tolerance (Kim *et al.*, 2010; Lee *et al.*, 2010; Lin *et al.*, 2011; Qin *et al.*, 2012). There was no obvious change to *CuZnSOD* expression in response to drought or re-watering treatments; however, *CuZnSOD* expression was elevated somewhat in ABI5 and RAVxABI5 stacked lines subjected to drought stress and recovery (Figure 4; lanes 9 and 10 vs. lane 12 and lanes 15–17 compared to lane 18), supporting a synergistic effect of these TFs in stress control through ROS scavenging.

Glutathione S-transferases (GSTs) play roles in normal cellular metabolism, oxidative stress response and detoxification of a wide variety of xenobiotic compounds (Apel and Hirt, 2004; Dixon and Edwards, 2010; Marrs, 1996; Sheehan *et al.*, 2001). The *GhGST* transcript was up-regulated approximately twofold in

response to drought in wild type (Figure 4). Furthermore, *GST* expression was elevated in response to re-watering compared to drought, suggesting a function in drought recovery in cotton. *GhGST* expression was higher in ABI5 and RAV1xABI5 overexpressing lines under WW and drought conditions compared to respective treatments of wild type (Figure 4). Remarkably, *GhGST* expression was higher in all the transgenic lines and stacked crosses during recovery from drought stress. These results support the hypothesis that faster recovery of photosynthetic capacity in transgenic cotton (Data S1) was due to higher ROS scavenging.

P5CS catalyses the rate-limiting step of proline biosynthesis and is required for ROS reductions in response to drought, salinity and ABA in Arabidopsis (Gu *et al.*, 2010; Qin *et al.*, 2012; Székely *et al.*, 2008). All the transgenic lines had reduced *GhP5CS* transcript abundances in comparison with wild type (Figure 4). *GhP5CS* was reduced approximately 70% in wild type under imposed drought. Interestingly, RAV1xABI5 stacked line accumulated more *GhP5CS* transcript compared with wild type under drought. All the transgenic lines including stacked crosses showed 1.5- to twofold higher transcript levels for *GhRAV*, *GhGST* and *GhP5CS* and lower stress marker *GhAdhA* at the recovery stage (Figure 4; lanes 13–18), supporting the notion of a 'less-stressed phenotype.'

Discussion

Overexpression of ABA-associated TFs such as nuclear factor-Y (NF-Y) can confer drought tolerance (Han *et al.*, 2013; Li *et al.*, 2008; Nelson *et al.*, 2007) and is a focal point for the development of the next generation of drought-tolerant crops. AtABI5 and AtRAV transgenic cotton exhibited higher or on par yields under WW greenhouse conditions and under drought stress in the field (Figure 2), reduced the inhibition of photosynthesis and improved WUE in response to imposed drought (Figure 3; Data S1), and enhanced carbon partitioned into leaves (Table 1), roots (Table 2) and stem (Tables S2, S3). Similar results have been reported recently for bZIPs, ABI3, NF-Ys, MYBs, AP2, WRKY and Enhanced Drought Tolerance/HOMEODOMAIN GLABROUS11 (EDT1/HDG11) classes of TFs (Abdeen *et al.*, 2010; Kumimoto *et al.*, 2013; Li *et al.*, 2013a; Liu and Howell, 2010; Ni *et al.*, 2013; Seo *et al.*, 2009; Yotsui *et al.*, 2013; Yu *et al.*, 2008, 2013; Zhang *et al.*, 2005), underscoring that there are many possible modes of TF engineering for vegetative drought stress tolerance. Remarkably, the stacked lines of RAVxABI5 exhibited synergistic effects on maintaining ***A*** in response to imposed drought (Figure 3a) and concomitant adaptation to higher WUE by decreases in stomatal conductance G_s, which translated into the generation of greater root mass (e.g. RAV2xABI5$^{13\text{-}4\text{-}1}$; Table 2). Both RAV1xABI5 crosses showed synergistic increases in stem weight (Table S2) and intrinsic WUE (Figure 3c), reminiscent of the drought resistance traits seen in drought-tolerant species such as Craterostigma, drought-tolerant wheat cultivars (Gupta *et al.*, 2011) and transgenic wheat constitutively overexpressing TaNF-YB3 (Stephenson *et al.*, 2011). A recent report also described overexpression phenotypes of a soybean RAV homologue in tobacco that largely resemble our cotton phenotypes of increased longevity and delayed flowering, increased lateral branching, however reduced root growth (Zhao *et al.*, 2012).

Increased root mass at later stages of vegetative development under WW conditions might not benefit crops because assimilate deposition would generate root biomass unnecessarily. However, increased sink strength resulting in bigger root systems under water deficit would help plants maintain higher turgor pressure, resulting in higher photosynthesis. Deeper root growth into moist soil is a second line of defence against drought (Pace *et al.*, 1999; Taiz and Zeiger, 2010). The transgenic cotton lines generated bigger root systems (Table 2; Figure S6) under greenhouse drought conditions. Enhanced root growth generates a competition for assimilates between roots and fruits. RAV and ABI5 transgenic cotton lines showed higher yields in the greenhouse compared to wild type (Figure S2A), suggesting very high WUE and possibly increased sink strength in bolls, which is currently under investigation in the field.

Use of ethylene (ethephon) is common in production agriculture for enhancing root growth. We speculate increased root growth observed in RAV/ABI5 transgenic cotton might be a result of ABA and ethylene crosstalk that alters sink strength. RAV TFs have also been described as ethylene response DNA-binding factors (EDFs) (RAV1 = EDF4; RAV2 = EDF2 and RAV2L = EDF1) and are ethylene inducible (Alonso and Stepanova, 2004). Previous characterization of RAV functions in brassinosteroids (Hu *et al.*, 2004), ROS scavenging (Lee *et al.*, 2010), ethylene response (Alonso and Stepanova, 2004), suppression of RNA silencing by viruses (Endres *et al.*, 2010), control of flowering time (Castillejo and Pelaz, 2008; Mutasa-Göttgens *et al.*, 2012; Osnato *et al.*, 2012), cytokinin signalling (Zhao *et al.*, 2012) and ABA signalling (present study) suggest that RAVs function as nodes in a crosstalk network, consistent with our unexpected observation (Figure 1c) that RAV2 and VP1 synergize in ABA-inducible gene expression, whereas VP1 and bZIPs are known to interact physically to transactivate ABA-inducible promoters (Finkelstein *et al.*, 2005). Recent reports on transcript profiling of the *Lignon lintless-1* or *fuzzless-lintless* (*fl*) mutants of upland cotton show associations between fibre elongation and hormone pathways, especially ethylene biosynthesis and differential expression of AP2/ethylene and stress response TFs (Gilbert *et al.*, 2013; Padmalatha *et al.*, 2012), consistent with our results and known functions of AtABI5 and AtRAVs. Transcriptome profiling or CHIP-Seq of the cotton transgenics may shed some light on the issues of direct versus indirect/hierarchical interactions of RAVs with ABI5 and each other, and their target genes impacting agronomic traits such as assimilation under stress, yields and fibre quality.

Altered carbon partitioning in the transgenic cotton lines resulted in bigger root systems and more leaf area, which if mechanistically conserved across species may facilitate engineering of crops to challenging environments and improve yields. Plants subjected to drought tend to reduce their internode length and become stunted. Remarkably, the cotton transgenics had longer internode lengths and concomitant increases in stem, leaf and total biomass (Table 1, Tables S1–S3; Figure S5) under water-deficit conditions in the greenhouse and field, and we interpret these traits as a 'less-stressed phenotype'.

Oxidoreductases play significant roles in response to biotic and abiotic stresses (Jacquot *et al.*, 2009; Reddy *et al.*, 2007). Lipid peroxidation in *CaRAV1*- and/or *CaOXR1*-silenced plants correlated with decreased tolerance to high salinity and drought (Lee *et al.*, 2010). CaOXR1 positively controls CaRAV1-mediated plant defence during biotic and abiotic stresses (Lee *et al.*, 2010). Importantly, RAV1xABI5 stacked transgenic cotton lines had approximately twofold higher transcript levels of *CuZnSOD* (Figure 4) under severe drought conditions and after re-watering. ABI5 overexpressing cotton also showed the same trend, emphasizing the synergistic effect of RAV1xABI5 in combating drought stress, analogous to our observations for transient gene induction (Figure 1). Higher *GST* transcript levels in RAV1xABI5

under imposed drought and a high level in all transgenics during stress recovery (Figure 4) suggest the 'less-stressed' phenotype is due to combating stress through increased levels of ROS scavengers. Similar results were recently reported for Arabidopsis expressing a peanut ABI5 homologue (Li *et al.*, 2013b). Reduced *AdhA* transcript in transgenics (and synergistic reductions in RAV1xABI5) under WW, drought and recovery conditions (Figure 4) supports the hypothesis of a less-stressed phenotype for ABI5 and RAV overexpressing cotton. In drought experiments, the transgenic plants were less wilty and leaves remained turgid due to increased water availability from bigger root systems (Table 2, Figure S6) and possibly osmotic adjustment (e.g. proline accumulation) mediated by twofold higher *GhP5CS* during stress recovery (Figure 4). Maintaining turgor enables the continuation of cell elongation and facilitates higher stomatal conductance at lower water potential (Taiz and Zeiger, 2010), observed in transgenic cotton as higher photosynthesis under drought and faster recovery (Figure 3a).

Our results with transgenic cotton overexpressing ABI5 and RAV TFs show drought resistance in photosynthesis and traits of drought avoidance (bigger root and leaf systems) and tolerance (manifest as longer internode length and higher stem weight) that may lead to better establishment under limited water conditions due to synergy with endogenous GhRAV and enhanced antioxidant and osmolyte synthesis. Higher ***A*** in ABI5/RAV transgenic cotton begs the question whether the most economically important sink (the developing bolls) exhibit gains in strength that could contribute to observed improved yields (Figure S2) or enhance fibre quality and seed traits such as oil and protein content. Better fibre quality under limited irrigation is a key trait for cotton producers, where staple prices are discounted because of immature or coarse fibres that result in poor yarn spinning performance. AtABI5 and AtRAV overexpressing transgenic cotton and potentially other crops could impact sustainable agriculture under limited irrigation and dry land farming, the ultimate consequence of continued depletion of the southern Ogallala Aquifer, the source of water for one-third of all US cotton production.

Experimental procedures

DNA constructs for transient gene expression

pBM207 contains the 650-bp *Triticum aestivum Early-Methionine-labelled* (*Em*) promoter driving *uidA* (*E. coli* β-glucuronidase, GUS) expression. pAHC18 contains the 2.0-kbp *Zea mays Ubiquitin* (*Ubi*) promoter driving *Photinus pyralis* luciferase (LUC) (Bruce and Quail, 1990). The *Viviparous-1* (VP1) cDNA effector driven by the *35S* promoter is pCR349.13s (Hill *et al.*, 1996). Plasmid p701-RAV2 is the *Ubi* promoter driving full-length cDNA clone U09382/At1 g68840/RAV2 (Yamada *et al.*, 2003) and was constructed using the Cre-lox recombination system of bacteriophage P1 (Liu *et al.*, 1998) in pCR701 as described (Jia *et al.*, 2009). Plasmids *Ubi*:ABI5 and *Ubi*:ABF3 were as previously described (Finkelstein *et al.*, 2005). Plasmid pG2 encodes the *35S*-maize *C4 pyruvate-orthophosphate dikinase* basal promoter chimera (*35S-Ppdk*) driving the coding region of the Arabidopsis *abi1-1* dominant-negative G180D mutant allele (Sheen, 1998). Plasmid pG1 is the same as pG2 except it encodes a G174D site-directed 'null' mutation that abolishes phosphatase activity (Sheen, 1998). Plasmid pDirect2.6 contains the *Ubi* promoter alone in reverse orientation used as control to balance input DNAs. Plasmids were propagated in *E. coli* DH5α, TOP-10 or GC10 cells (Invitrogen, Carlsbad, CA) and prepared by CsCl density gradient ultracentrifugation (Ausubel *et al.*, 1995).

Protoplasts

Zea mays seeds genotype FR37cms_X_FR49 (Illinois Foundation Seed, Champaign, IL) were imbibed in water overnight and sown in a 1:1 vermiculite/peat moss mix. Seeds were germinated in constant incandescent light for 4 days at 23 °C and moved to a dark growth chamber when coleoptiles emerged. Protoplast isolation was according to Sheen (2001) with modifications (Jia *et al.*, 2009). 50 000 protoplasts per electroporation sample in 300 μL were mixed with DNAs and transferred to prechilled 0.4-mm gap cuvettes (Bio-Rad, Hercules, CA). After 10 min on ice, samples were electroporated (400 V, 200 μF; two pulses) with a BTX Electro Cell Manipulator 630 (Gentronics, San Diego, CA) and incubated on ice for 10 min. Protoplasts were then split into two aliquots in microfuge tubes and incubated with either wash solution only, or 100 μM ABA in wash solution. After 16-h incubation in the dark, cells were pelleted by centrifuging at 800 rpm for three min, and 250 μL 1× Reporter Lysis Buffer (Promega, Madison, WI) was added to the pellet and thoroughly vortexed. The lysate was centrifuged at 10 000 × ***g*** for three min and the supernatant removed to a fresh tube for reporter enzyme assays. Protein was quantified using Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

Immunoblotting

SDS-PAGE was as described (Ausubel *et al.*, 1995; Towbin *et al.*, 1979), loading equal amounts (5 μg/lane) of protein along with prestained low-range SDS-PAGE standards (Bio-Rad). The gel was electroblotted to Immobilon-P PVDF transfer membrane (Millipore, Billerica, MA) using the MiniBlot Module (Thermo EC, Waltham, MA). Immunoblotting was performed according to the manufacturer's instructions using ECL Advance Western Blotting Detection Kit (Amersham Pharmacia/GE Healthcare Bio-Sciences, Pittsburgh, PA). The primary mouse anti-HA monoclonal antibody (clone HA-7, Sigma, St. Louis, MO) was used at 1:5000 dilution. The secondary was goat anti-mouse IgG_1-conjugated horseradish peroxidase (sc-2060; 1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA).

Reporter enzyme assays

Ten microlitres of sample extract was mixed with 50 μL of luciferase substrate (Promega) and luciferase activity measured on a Zylux FB15 luminometer (Fisher Scientific, Pittsburgh, PA). GUS activities of 40 μL aliquots of samples (four timepoints) were determined (Jefferson, 1987) using 4-methylumbelliferone glucuronide (MUG; Rose Scientific, Edmonton, Canada) as substrate on a Biotek (Winooski, VT) Synergy HT microplate fluorimeter. The relative reporter gene activity was represented as the ratio of GUS to LUC activities, expressed in relative units (or μmoles) of 4-MU/40 μL extract/h and photons/10 μL extract/min, respectively.

Generation of recombinant vectors for transformation of cotton

We employed available pUNI51-derivative full-length cDNA clones of Arabidopsis RAV family members RAV1 (U11954), RAV2 (U09382), RAV2L (U19336) and ABI5 (U85657; Arabidopsis Biological Resource Center, Ohio State University, http://abrc.osu.edu/) to recombine pKYLX-myc9-loxP binary acceptor vector (Guo and Ecker, 2003) in the presence of cre recombinase enzyme. For recombination, in a 20-μL reaction volume, 500 ng of each of

acceptor and donor vector DNAs was mixed with 2 μL 10 × recombination buffer (New England Biolabs; www.neb.com) and 2 μL GST::CRE recombinase (Jia *et al.*, 2009) and incubated at 37°C for 20 min. The DNAs were precipitated with EtOH, the pellet dissolved in 10 μL water, and the DNA measured by a Nanodrop spectrophotometer (Wilmington DE). About 200 ng of DNA products were electroporated into electrocompetent *pir*$^{-}$ *E. coli* GC10 cells (Invitrogen), and the bacterial colonies carrying recombinant fusion plasmid were selected on kanamycin-containing LB plates. The candidate transformation-ready constructs were restriction digested and were confirmed to be comprised of a dimer of one acceptor and one pUNI donor plasmid. Plasmids were electroporated into electrocompetent *Agrobacterium tumefaciens* strain GV3101.

Deficit irrigation treatments and gas-exchange measurements

Field trials were conducted at the TTU New Deal Farm south plot with subsurface drip irrigation under USDA-APHIS Permit No.11-097-106n. Sowing was carried out on 7 June 2011 for 17 transgenic lines and three Coker check groups in a randomized block design with a zone subjected to DI (1/4 acre-inches water/day until flowering stage [day 42 after sowing (DAS)] for DI) as well as a WW control treatment zone. All lines tested in the field condition were homozygous (confirmed by PCR sampling) and were planted in paired rows with other commercial genotypes included as needed to fill up the plot to minimize border effects. There were eight rows (40″ spacing) of approximately 140 feet for each watering treatment zone, giving an overall field plot of approximately 0.5 acre. Mechanical sowing was at the rate of four and one-half seeds per foot in eight-feet-long subplots. For greenhouse experiments, potting mix, field soil and sand were mixed in 3:1:1 volume proportions, respectively. Photosynthesis parameters were measured using a Licor-6400 XT (LI-COR Biosciences, Lincoln, NE) and taken in representative lines of all the transgenics starting 1 week after and until the sixth week of the DI treatment. For greenhouse experiments, the last watering was given on 24 DAS, and measurements were commenced on 7 days after withholding water (7D-drt) and continued until 5 days after re-watering (5D-Rec) (10 days in total). Measurements were restricted to 10 am–1 pm when temperatures were not extreme, and plants from all lines were measured within half an hour using expanded source leaves (4th or 5th leaf from apical meristem). Greenhouse plants were not watered until more than 80% of wild-type control plants did not show evening recovery from afternoon wilt. Several cycles of this treatment were repeated until 90 DAS. Tissue for stress marker gene analysis was collected from six individual greenhouse plants of each line for WW nonstressed condition (24 DAS), drought treatment (11 days of no watering; 35 DAS) and recovery (overnight recovery from drought stress after re-watering).

Biomass assays

Owing to the labour-intensive nature of hand harvesting, we measured fibre yields in 1-metre rows (in triplicate) from the interior regions of field plots (to discount border effects). For greenhouse studies, all the plant parts (root, stem, leaves and fruits) were collected separately. Leaves were detached (leaving the petioles intact on the stem) and kept in ziplock bags at 4 °C. Leaf area was measured using a LI-3100C Portable Leaf Area Meter. Fruits (flowers, immature and maturing bolls) and stems were detached and stored in paper bags in the greenhouse. After removing fruit, leaves and stems, the pots were dipped in a washtub filled with water until saturated and manipulated to release the potting soil/earth/sand mixture from intact roots. After the roots were processed as a ball without any adhering soil, they were washed in several changes of fresh water and stored in ziplock bags until imaged. All the plant parts were dried in an oven at 74 °C for 72 h and weighed. To obtain the weight of lateral roots, the whole dried roots were weighed, all the lateral roots were removed, and then the bare taproot was weighed. The difference gave the weight of lateral roots, shown in Results.

Semi-quantitative RT–PCR

Total RNA was extracted using Spectrum Plant RNA Mini Kit (Sigma-Aldrich, St. Louis, MO). Sigma on-column DNase1 digestion was used to remove DNA contamination in extracted RNA. Two micrograms of RNA was reverse transcribed by M-MLV reverse transcriptase (Promega) with Anchored Oligo-dT (Thermo, Surrey, UK). 0.5 μL of cDNA template was used for a 25-μL PCR. Gene-specific primers (Table S4) for *AtRAV1*, *AtRAV2*, *AtRAV2L* and *AtABI5* were used to amplify (35 cycles) the cDNA from transgenic lines. *GhUBQ1*-specific primers were used as an internal control.

RNA blot hybridization assay

Ten micrograms of total RNA per sample was resolved on 1.2% denaturing agarose gel and blotted onto a Hybond-N+ membrane (GE Healthcare, Piscataway, NJ). An RNA molecular weight marker lane was included to estimate mRNA transcript sizes (Ambion Millenium Marker, GE Healthcare). Primers (Table S4) were designed based on BLAST results from NCBI plant EST database, and cDNA was amplified from reverse-transcribed Coker312 RNA. The PCR products were gel-purified and used as template for random-primed synthesis (Takara, Shiga, Japan) of radioactive probes with [α^{32}P]-dCTP (PerkinElmer, Waltham, MA). PerfectHyb Plus hybridization buffer (Sigma) was used according to the manufacturer's instructions. Autoradiography was with storage phosphor screen (GE Healthcare) scanned with Storm 860 PhosphorImager (GE Healthcare). Ethidium bromide-stained total RNA samples were quantified from gel images using ImageJ software (imagej.nih.gov/ij/download). The RNA blot band intensity was quantified using ImageQuant TL software (v2003, GE Healthcare). The ratio of ImageQuant to ImageJ values gives normalized transcript quantity for relative comparisons.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 RNA blots of independent transgenic cotton lines showing over-expression of transcripts for Arabidopsis transgenes compared to wild type.

Figure S2 (A) Cotton seed plus fibre yield of transgenics compared to wild type under WW greenhouse conditions, (B) under WW and DI conditions in the field (2011).

Figure S3 Severe wilting of DI Coker312 (left) compared to DI RAV1xABI5 (right).

Figure S4 Deficit irrigation treatment effect on biomass accumulation.

Figure S5 Total plant height after 90 days of DI in several individual wild-type Coker312 plants (left) and compared to RAV2 transgenic cotton individuals (right; event RAV2$^{24\text{-}8\text{-}1}$), which have on average 17% longer internodes.

Figure S6 RAV and/or ABI5 transgenic cotton generates greater root biomass.

Table S1 Internode length (cm) in transgenic cotton lines subjected to 90 days of DI in greenhouse conditions.

Table S2 Average stem dry mass (g/plant) in wild type and transgenic cotton lines subjected to 90 days of DI in greenhouse conditions.

Table S3 Total dry mass (g/plant) in transgenic cotton lines subjected to 90 days of DI in greenhouse conditions.

Table S4 List of primer pairs used in RT-PCR and for amplifying probes for Northern blot assay from cDNA library of cotton.

Data S1 Photosynthesis parameters for field and greenhouse trials of RAV and ABI5 transgenics.