Title

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Running head: AtRAV1 and AtABI5 affect stomata in cotton

Title of article: Characterization of transgenic cotton (*Gossypium hirsutum* L.) over-expressing *Arabidopsis thaliana* Related to ABA-Insensitive3(ABI3)/Vivparous1 (AtRAV1) and AtABI5 transcription factors: improved water use efficiency through altered guard cell physiology

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Potential Summary Text, for the Table of Contents: Cotton is a major cash crop with farm gate value estimated at > $40b globally/yr and utilizing ~2.5% of the world's arable land. We characterize mechanisms of improved water use efficiencies via activities of two transcription factor transgene products. These transgene products affect stress hormone responses and impact guard cell development, and they also affect stomatal density and function. Our ~~results~~ analyses of multiple tissues frame a systems approach to understand the complexity associated with transcription factor effectors and provide insights~~solution~~s to improved engineering of drought stress adaptation of crop plants.

Abstract

We investigated select transgenic lines of *Gossypium hirsutum* that over-express AtRAV1 or AtABI5 transcription factors. The hypothesis is that these lines ~~, previously characterized for their molecular and physiological phenotypes,~~ have enhanced responses to abscisic acid (ABA)~~,~~ resulting in greater water use efficiencies (WUE) ~~under drought stress~~. We measured leaf surface temperatures (LST), stomatal density, absolute and relative sizes of guard cell apertures, and ABA concentrations in cotyledons. We ~~also~~ characterized transgene protein expression and activities in transient assays in *Nicotiana benthamiana*, in transgenic cotton seeds by immunoblot and by ~~, activities of transgenes in leaves assessed by~~ endogenous expression in leaves of an ~~positive~~ effector of ABA responses (*GhDREB*)~~, and stomatal conductance and photosynthesis rates in greenhouse-grown plants~~. AtRAV1$^{1\text{-}1\text{-}5}$ and AtABI5$^{1\text{-}1\text{-}1}$ over-expression lines had trends of lower levels of ABA in well-watered and drought-stressed cotyledons, and all events tested had ~~significantly~~ higher leaf stomatal conductance and photosynthesis rates under drought in greenhouse, and lower LSTs than control Coker 312 under drought stress conditions. Notably, AtRAV1$^{1\text{-}1\text{-}5}$ cotyledons had significantly higher stomatal densities and 26% smaller guard cell apertures than Coker312 under drought stress, providing a plausible explanation why LSTs across lines were lower concordant with smaller stomatal apertures. ~~Results suggest smaller guard cell pores and greater stomatal densities~~These traits may contribute to intrinsic WUE and assimilate traits of larger leaf areas and longer boll fibers previously shown in these and several independent AtRAV1 and AtABI5 events in the greenhouse and field. These results are consistent with the hypothesis that over-expression of AtRAV1 results in an ABA-hypersensitive phenotype manifest as reduced expression of endogenous *GhDREB* effector, and lower levels of endogenous ABA in cotyledons associated with greater reductions in pore apertures during stress and increased stomatal density.

Keywords: abscisic acid; photosynthesis; transpiration; dicotyledon; stomatal conductance; drought tolerance

Introduction

There is a growing need for more food and fiber - this demand clearly justifies why crop engineering is important. It is also the case that climate change will increase the frequency and severity of drought events, so crop engineering of drought tolerant crops is vital. Limited water availability is one of the major abiotic factors impacting crop production worldwide. Significant efforts have been taken to enhance drought tolerance (high water use efficiency [WUE], osmotic adjustment) and avoidance (early flowering, increased root biomass) traits (Heschel and Riginos 2005; McCann and Huang 2008) in crop plants through two main approaches: 1) traditional plant breeding at the scale of whole plants (so called 'top-down' approaches), and 2) advanced bioengineering at the scale of individual genes (so called 'bottom-up' approaches) (Sinclair 2011). While advances have been made in the development of drought-tolerant cultivars, knowledge about the metabolic and physiological traits that functionally contribute to the drought-tolerant phenotypes remains limited. Understanding how genotypes produce a given phenotype will contribute towards closing the gap between the top-down and bottom-up approaches (Miflin 2000), and may facilitate development of efficient protocols for screening candidate drought-tolerant cultivars (Fiorani and Schurr 2013).

Genomic and genetic analyses primarily of the model plant *Arabidopsis thaliana* (L. Heynh.) have elucidated the biosynthetic and signal transduction pathways of abscisic acid (ABA) and its roles in stress response (reviewed in Rock *et al.* 2010; Cutler *et al.* 2011). Altered expression of several different ABA effectors (e.g. ENHANCED RESPONSE TO ABA1 [ERA1/Farnesyl Transferase beta subunit], AtNuclear Factor Y-B1, AtNFY-A5, ABA3/LOW OSMOTIC STRESS RESPONSE5/[LOS5]) have been shown to enhance drought tolerance in canola (*Brassica napus* L.), corn (*Zea mays* L.), and rice (*Oryza sativa* L.) (Wang *et al.* 2005; Nelson *et al.* 2007; Xiao *et al.* 2008; Li *et al.* 2008). Similarly, transgenic cotton (*Gossypium hirsutum* L.) lines have been generated that over-express the basic leucine zipper domain transcription factor AtABA-INSENSITIVE5 (Brocard *et al.* 2002) and Basic3/APETALA2-domain transcription factors of the Related to ABI3/Viviparous1 (RAV) clade AtRAV1, AtRAV2/TEMPRANILLO2, and AtRAV2L/TEMPRANILLO1, the latter of which has been shown in *Arabidopsis* to regulate flowering time and stomatal aperture by repression of FLOWERING LOCUS T (FT)(Ando *et al.* 2013; Castillejo and Pelaz, 2008; Kinoshita *et al.* 2011; Lu *et al.* 2014; Sgamma *et al.* 2014) and stress and defense pathways in different species

(Endres *et al.* 2010; Li *et al.* 2011). The resultant cotton transgenics expressing AtRAV1, AtRAV2, and AtABI5 show genetic, morphological and agronomic responses, including altered environment-regulated and fiber-associated gene expression, delayed flowering time, increased WUE, and greater root biomass and fiber length under drought irrigation stress in the greenhouse and field (Mittal *et al.* 2014, 2015). These results and others (Liang et al., 2016; Sohn *et al.* 2006; Tang *et al*. 2012; Xin and He 2013) suggest that over-expression of RAV and bZIP genes in cotton produces drought-avoidance and drought/salt tolerance phenotypes, but the molecular mechanisms underlying agronomic stress-response traits remain unclear.

In the present study, we characterized a subset of the AtRAV1 and AtABI5 over-expressing transgenic lines reported in Mittal *et al.* (2014, 2015). Two lines $RAV1^{1\text{-}1\text{-}5}$ and $RAV1^{13\text{-}7\text{-}1}$ were chosen based on prior characterization of their intermediate levels for physiological characters of *AtRAV1* overexpression in leaves, effects on larger leaf areas, increased intrinsic WUE under well-watered and drought conditions, and increased fiber lengths in the field (Mittal *et al.* 2014, 2015). Our goal was to further characterize representative cases for the role of AtABI5 and AtRAV1 overexpression on a known drought tolerance mechanism: ABA-sensitivity of guard cells. ABA is an important regulator of plant responses to drought-stress and is commonly observed at increased levels in drought-stressed plants (Rock *et al.* 2010; Zeevaart 1980). One such response governed by ABA is the turgidity of guard cells (Kim *et al.* 2010), which determines the size of guard cell pores (i.e., stomatal aperture). Guard cell pores are the gateway for both CO_2 intake and water loss (transpiration) in plants. In conjunction with ABA measurements, we measured the areas (μm^2) of cotyledonary leaf guard cells and their pores, leaf surface temperatures (LST), and photosynthetic parameters and stomatal conductance in five-week-old greenhouse-grown leaves. The density and relative size of guard cell pores (pore area/guard cell area) were calculated as additional metrics to evaluate the potential effects of the transgenes on stomatal development and response. We found that, consistent with the "less stressed" and drought avoidance phenotypes of the transgenic lines previously characterized, the $AtRAV1^{1\text{-}1\text{-}5}$ and $AtABI5^{1\text{-}1\text{-}1}$ overexpression lines had greater stomatal densities at the cotyledonary stage and under drought stress showed relatively lower cotyledonary leaf temperature rise, lower ABA levels during drought that increased during recovery, and smaller stomatal apertures, allowing inference of a hypersensitivity to ABA and probable homeostatic feedback adaptation of stomata in AtRAV1 over-expressing lines, supported by concordant

changes in endogenous ABA effector gene expression in transient assays and transgenic leaves ~~*GhDREB*~~ as markers of ABA sensitivity.

Materials and Methods

Germplasm

This study originated as an investigation of the effects of drought stress and genotype on the feeding and fitness of Western Flower Thrips, *Frankliniella occidentalis* (Pergande) (Fiene 2012), an intracellular feeding herbivore that infects and damages cotton at predominantly the seedling stage (Fiene *et al.* 2013). Thus cotyledons were studied initially for drought stress by genotype interactions of select lines that showed promising preliminary results. The three transgenic cotton (*Gossypium hirsutum* L.) lines selected were previously generated by transformation of cotton cultivar 'Coker 312' (wild-type; USDA Germplasm Resources Information Network #PI529278; Plant Variety Protection #7200100), which undergoes facile regeneration from somatic embryos. The specific transgenes with associated event numbers in superscript were the following: RAV1$^{1\text{-}1\text{-}5}$, RAV1$^{13\text{-}7\text{-}2}$, ABI5$^{1\text{-}1\text{-}1}$, and ABI5$^{13\text{-}4\text{-}1}$ (Mittal *et al.* 2014, 2015). Effector transgene T-DNA constructs have an N-terminal c-myc epitope tag for facile immunodetection (Guo and Ecker, 2003). All cotton plants (n = 21-24 per cotton line) were seeded individually in 400 mL pots with Metro-mix 900 soil and simultaneously cultivated in a room (2.25m x 2.75m x 2.25m) at 34.7^{o} C under continuous light (13.1±5.2 μmol m^{-2} s^{-1}) for 13 days. Plants emerged from the soil four days after planting (DAP). Plants with fully expanded cotyledons were treated five DAP (except for photosynthesis measurements, see below) by withholding water, resulting in a progressive drought stress (DS) for seven days followed by one day of drought recovery after re-watering with 125 mL of water on 12-DAP which marked the start of the drought-recovery period. One day later (13 DAP), cotyledons were harvested from plants.

Leaf surface temperatures

We measured LST from days four to seven of drought stress using a Fluke 62 IR thermometer (http://en-us.fluke.com) to validate plant responses consistent with drought stress; namely a reduction in transpiration rate via stomatal closure resulting in elevated LST. Three subsamples of LST were collected from each cotyledonary leaf in rapid succession from the

adaxial surface ~two cm from the petiole. The six subsamples per plant (two cotyledons x three subsamples) were averaged to provide a single data-point per plant (n = 7-12). The distance (roughly 2.5 cm) and angle (90 degrees) between the cotyledonary leaf and IR thermometer was constant, and each plant was assayed in the same part of the room in order to minimize variation (Okono 2011). As the drought treatment progressed some of the cotyledonary leaves drooped (became less turgid), and so a pen was used to lift the leaf to orient it horizontally; after 30s of acclimation LSTs were measured. Pre-drought (9 DAP) LST did not meet assumptions of normality and a generalized linear model (Gaussian error structure) was used with an F test. LSTs collected during the drought-stress period (10-12 DAP) and recovery (13 DAP) met assumptions of normality, and a repeated measure ANOVA was used with an error term indicating DAP nested within plant in the former, and an ANOVA was used in the latter (R Development Core Team 2009).

Immunoblotting

Total protein from seeds of two ABI5 lines (1-1-1 and 13-4-1) and RAV1$^{1\text{-}1\text{-}5}$ line, plus wild type Coker 312 cotton was extracted with protein lysis buffer (2-mercaptoethanol, EDTA, Triton-X, Sodium Phosphate Buffer, PMSF) and quantified using Bradford Coomassie assay (Pierce; www.piercenet.com). Briefly, 10 µg of total protein was loaded onto a mini vertical SDS-PAGE gel (Thermo Fisher Scientific; www.thermofisher.com) (Ausubel *et al.* 1995) and blotted on to Immobilon-P PVDF transfer membrane (Millipore; www.millipore.com). Monoclonal mouse anti c-myc primary antibody (Pierce, 1:2000 dilution) was used to detect the protein expressed by the transgene. Horse radish peroxidase-labeled anti mouse secondary antibody (1:20,000 dilution) was used to bind primary antibody. To validate lane loadings, blots were re-probed with mouse monoclonal anti-Ubiquitin primary antibody (PD41, Abcam. http://www.abcam.com). ECL Advance chemiluminescence detection method (Amersham Biosciences; www.gehealthcare.com) was used to detect protein on X-ray film and on a Biorad (Hercules, CA; www.bio-rad.com) Chemidoc MP system controlled by Image Lab™ software.

As a positive control for immunoblotting experiments and to assay transgene activities on endogenous ABA responses, transient expression of AtRAV1 and AtABI5 in *Nicotiana benthamiana* leaves was performed by infiltration of a solution of *Agrobacterium tumifaciens* strain GV2260 (Llave *et al.* 2000) harboring the same T-DNA vectors (Guo and Ecker, 2003;

Mittal *et al.* 2014) used to generate the stably transformed cotton lines ~~RAV1~~[1-1-5]. Plants were grown at 21°C with a 16 hr/8 hr, light/dark cycle for long day conditions for four weeks. 24 hours post-inoculation (one hour post-inoculation for ABA response), total proteins or RNAs were extracted from dissected leaf tissue and analyzed by ~~immuno~~blotting. Quantification of immunoblot band intensities was with Quantity One® software (BioRad), taking the average of five band slices of equal dimensions.

RNA blot analysis

RNA blot analysis was on six or 10 µg of total RNA extracted respectively from either *N. benthamiana* transiently transformed leaf disks, or six pooled leaves from individual greenhouse-grown transgenic lines, treated as well-watered, non-stressed condition ("WW"; 24 DAP), -drought treatment ("Drt"; 11 Days of no watering; 35 DAP), and -recovery (overnight recovery from drought stress after rewatering, "Rec"; 36 DAP). Total RNA was resolved on 1.2% denaturing agarose gel containing formaldehyde and blotted to a Hybond-N+ membrane (GE healthcare, Piscataway, NJ) according to the manufacturer's instructions. An RNA molecular weight marker lane was included to verify mRNA transcript size (Ambion Millenium Marker, cat# AM7151). Oligonucleotide primers were designed using primer 3 design (http://frodo.wi.mit.edu/) and/or 'Perlprimer' (http://perlprimer.sourceforge.net/) and synthesized commercially (Sigma, St. Louis MO). Primer sequences CAGCAAGCGGAGAGAAGCGAAA and TCGATGATTCCGATGATGAAGCA were used to PCR amplify a 362 bp cDNA amplicon corresponding to APETALA2/ethylene-responsive transcription factor *Dehydration Response Element-Binding protein-RAP2-1-like (GhDREB)* 866 nt mRNA (GenBankAF509502) using oligo dT-primed reverse-transcribed cotton leaf total RNA as template. Primers for *N. benthamiana* probes *LEA-5* (solgenomics.net annotation Niben101Scf19584g00018.1; GenBank Q39644) were forward TTGTTAGCAGGCGTGGGTAT, and reverse CTCTCGCTCTTGTTGGGTTC as described (Huo *et al.*, 2016). Primers for *ERD10C* (Niben101Scf05385g04007.1, GenBank ADQ73987.1) were forward GATGAGGAGGAAGAAATAGG and reverse CTTCAGTCTTTGAGTGGTAT. Probes were synthesized using Random Primer DNA Labeling Kit Ver.2 (TAKARA, Shiga, Japan) with [α-^{32}P]-dCTP (PerkinElmer, Waltham, MA). Hybridization was carried out with the PerfectHyb Plus hybridization buffer (SIGMA ALDRICH, Saint Louis, MO) according to the

manufacturer's instructions. A storage phosphor screen (GE Healthcare, Piscataway, NJ) was used for autoradiography and it was scanned using a Storm 860 PhosphorImager (GE Healthcare). For quantification of band intensities, ethidium-bromide stained ribosomal RNA was quantified as loading control using Quantity One® or ImageJ software (https://imagej.nih.gov/ij/) and RNA blot band intensity was quantified using ImageQuant TL software (v2003, GE Healthcare) and normalized relative to loadings to derive fold change by treatments.

LC-MS/MS analysis of plant hormones

Phytohormones and guard cell morphology were analyzed at peak drought (12 DAP) and drought recovery (13 DAP), and on each day three plants were harvested per treatment with one cotyledon analyzed for phytohormones and the other for guard cell morphology (see below). Fresh cotyledon tissue (80-160 mg) was collected from individual cotyledons and flash frozen in liquid N_2. The quantification of plant hormones was performed by the Proteomics and Mass Spectrometry Facility at the Donald Danforth Plant Science Center (St. Louis, MO, USA). The method is similar to Chen *et al.* (2009), but modified to include additional plant hormone species (Weber *et al*. 1997). Briefly, samples were ground in liquid N_2 and deuterated internal standards (10 mL of 2.5 mM) were added (Supplementary Table S1). Samples were extracted with 1.5 mL acetonitrile/methanol (1:1 v:v). After lyophilization, samples were resolubilized in 200 mL of 50% MeOH. For liquid-chromatography separation, a monolithic C_{18} column (Onyx, 4.6 mm x 100 mm, Phenomenex, CA, USA) with a guard cartridge was used at a flow rate of 1 mL min^{-1}. The gradient was from 40% solvent A (0.1% (v/v) acetic acid in MilliQ water), held for 2 min, to 100% solvent B (90% acetonitrile (v/v) with 0.1% acetic acid (v/v) in 5 min. The LC was held at 100% B for 3 min and then ramped back to initial conditions and re-equilibrated for an additional 2 min. To minimize variation from the autosampler, the sample loop was overfilled with 52 µL of sample and the sample storage temperature was set to 8°C. The LC-MS/MS system used was a Shimadzu LC system with LEAP CTC PAL autosampler coupled to an Applied Biosystems 4000 QTRAP mass spectrometer equipped with a TurboIon Spray electrospray ion source. Source parameters were set to: CUR: 25, GAS1: 50, GS2: 50 (arbitrary unit), CAD: high, IHE: on, TEM: 550°C, IS: -4500. Both quadruples (Q1 and Q3) were set to unit resolution. Analyst software (version 1.4.2) was used to control sample acquisition and data analysis. To maximize

sensitivity, ABA, salicylic acid, 12-oxo-phytodienoic acid, and jasmonic acid standard solutions were infused into the 4000 QTRAP with a syringe pump (Harvard 22) at 10 mL min^{-1} to select multiple reaction monitoring (MRM) transitions and optimize compound-dependent parameters for MRM detection (Supplemental Table S1).

ABA quantification

A dilution series of standards was prepared containing different concentrations of ABA and D-labeled ABA and SA (250 pmol/sample). Correction factors were obtained by adjusting the ratio of standard peak areas to that of internal standards in all samples. The peak areas of endogenous hormones were normalized with the corresponding internal standard and then calculated according to the standard curve. ABA is reported in ηg per mg plant fresh weight. H_2JA was also used for the quantification of JA-Ile because no D-standard for that compound is commercially available.

Guard cell morphology

Guard cell morphology was analyzed by a modified technique described by Travaglia *et al.* (2010). A layer of clear nail polish (nitrocellulose in ethyl acetate) was brushed on the abaxial side of a cotyledon, allowed to dry for 15s, and then carefully extracted with forceps and mounted on a microscope slide. The slide was examined using a standard compound microscope, and digital photomicrographs taken of the abaxial leaf impression. The photomicrographs were then imported into Image-J (Rasband, 1997-2012), and the numbers and area (μm^2) of guard cells (i.e., individual stoma) and their pore (i.e., aperture) were analyzed. Stomata measurements were chosen at random from those that could be clearly focused as to avoid distortion in the measurements. Because of variation in the clarity of individual guard cells in images, the number of guard cells per cotyledon characterized for aperture ranged from 15-20 stomata per cotyledon, and the total number of stomata counted per genotype was from 300 to 440, with 12 individual plants for each genotype sampled from seven to eight times. The area of guard cells and pores were summed to produce a single data point for that cotyledon, and then scaled to 15 to correct for the uneven number of guard cells collected per cotyledon. We also investigated the area of guard cell pores relative to the size of guard cells (pore area/guard cell areas) by dividing the total areas of 15 guard cells by the summed areas of the pores.

Root and shoot biomass, stomatal conductance and photosynthesis measurements

To further characterize the possible underlying mechanisms for apparent lower transpiration in RAV1$^{1\text{-}1\text{-}5}$, we analyzed the effects of drought stress on biomass accumulation, photosynthesis, and stomatal conductance in individual greenhouse-grown homozygous transgenic lines over-expressing AtABI5 or AtRAV1 using a Licor LI-6400XT Portable Photosynthesis System. Measurements were taken on near-fully-expanded leaves of five week-old plants for six days. Plants were grown in potting mix, field soil and sand (3:1:1) in 3.5 gallon pots to better imitate field conditions. Biomass of individual plants was determined by weighing oven-dried material harvested at 90 DAP.

Statistical analysis

To determine the effects of each transgenic event on responses to drought-stress and recovery, we conducted pair-wise analyses of Coker312 against each transgenic cotton line under specific treatment conditions. This was done using a two-tailed Student's *t*-test, assuming equal variance except for ABA quantifictation, where a one-tailed t-test was used on grounds prior work (Mittal *et al.* 2014, 2015) showed the test genotypes were drought tolerant/ABA hypersensitive.

Results

***Characterization of transgene protein accumulation in cotton seeds and endogenous ABA response marker expression in transient assays overexpressing* AtRAV1 *and* AtABI5**

Previous work showed high levels of mRNA expression in leaves and developing ovules for numerous independent *ABI5* and *RAV1* transgene events driven by the 35S promoter (Mittal *et al.* 2014, 2015), so we endeavored to validate transgene protein accumulation in the select events under study, which represented typical reproducible phenotypes described previously. We focused on seeds and cotyledons because our initial hypothesis was that AtABI5 would affect seed development/maturation. Figure 1 shows a slightly higher level (~5% by dosimetry) of c-myc: epitope-tagged :ABI5$^{1\text{-}1\text{-}1}$ expression compared to :ABI5$^{13\text{-}4\text{-}1}$ expression in protein extracts of transgenic T2 (homozygous, second generation) seeds. This observation is consistent with prior results from greenhouse and field that the AtABI5$^{1\text{-}1\text{-}1}$ event has more extreme phenotypic effects than AtABI5$^{13\text{-}4\text{-}1}$ for longer fiber lengths, larger leaf area, greater root

biomass, and higher WUE (Mittal *et al*., 2014, 2015). L~~A l~~ower~~, but still~~ detectable (inset arrow)~~,~~ levels of c-myc::RAV1$^{1\text{-}1\text{-}5}$ and c-myc::RAV1$^{13\text{-}4\text{-}1}$ expression were~~as~~ observed in transgenic seeds. The relatively higher levels of c-myc signal in RAV1$^{13\text{-}7\text{-}2}$ versus RAV1$^{1\text{-}1\text{-}5}$ seeds correlate with *AtRAV1* mRNA levels quantified by real-time-PCR in leaves from these transgenic lines from 51- 80 DAP and relatively increased effects of RAV1$^{13\text{-}7\text{-}2}$ overexpression on fiber length and delayed boll cracking in the field versus RAV1$^{1\text{-}1\text{-}5}$ under drought conditions (Mittal *et al*., 2015), and for intrinsic water use efficiency (Mittal *et al*., 2014). In order to verify the faint bands observed corresponded to bona fide c-myc::RAV1, a transient gene expression assay was performed and proteins extracted and immunoblotted after ~~7~~24 hr infiltration of *Nicotiana benthamiana* leaves with a culture *Agrobacterium tumifaciens* harboring the same vector construct used for stable transformation (Fig. 1A). High non-specific background signals using c-myc antibodies on leaf extracts of cotton precluded our efforts to convincingly quantify expression of transgene effectors (data not shown; see Fig. 1A and prior results in maize (Jia *et al*., 2009)). Feng *et al.* (2014) showed that the transcription of *RAV1* in Arabidopsis is very low in imbibed seeds, consistent with our result.

In order to assess the function of overexpressed heterologous transcription factors associated with stress adaptation, we analyzed by RNA blot the expression of endogenous ABA response markers *LEA-5* and *ERD10C* (Hou *et al*., 2016) in transient transformation assays with *N. benthamiana*. Fig. 1B-D shows that overexpression for 24 hours of AtRAV1, and to a lesser extent AtABI5, in leaves resulted in two-fold trans-activation of endogenous ABA response marker genes. These results are consistent with prior results (Mittal *et al*., 2014) which showed that endogenous stress response markers *Responsive to ABA18* (Gh*RAB18*), Gh*RAV*, Gh*CuZnSOD*, Gh*GST*, and Gh*P5CS* mRNAs were similarly elevated in leaves of the stable cotton transgenic lines, whereas Gh*AdhA* was expressed correspondingly lower than control during drought recovery. Correlations between higher stress marker expression, photosynthesis, and WUE support that the transgenic lines have a 'less-stressed' or stress-adapted phenotype via increased ROS scavenging. Taken together the evidence supports that relative expression levels of effector transgenes in seeds generally correlate with relative expressions of mRNAs in other vegetative tissues associated with physiological effects of transgene expression.

Drought stress results in lower ABA accumulations in* RAV1$^{1\text{-}1\text{-}5}$ *and* ABI5$^{1\text{-}1\text{-}1}$ *seedlings, and higher accumulations during recovery

Previous evidence showed that AtRAV1- and AtABI5-over-expressing cotton had "less stressed" and drought avoidance phenotypes under imposed drought (Mittal *et al.* 2014, 2015), therefore we measured ABA levels in cotyledons in response to drought stress to independently address the hypothesis that ABA signaling processes may be impacted in these lines. On day seven under drought-stress, control Coker312 plants showed a 'wilty' phenotype and had ABA concentrations that were about three-fold higher than in well-watered plants ($P < 0.05$, Fig. 2A), demonstrating the efficacy of the drought treatments. During drought-recovery, ABA levels in well-watered samples were higher than samples harvested the day before, an observation that held across all genotypes which suggest an uncontrolled or non-specific environmental effect not observed in the drought-stressed samples. However, ABA levels in control Coker 312 and ABI5$^{13\text{-}4\text{-}1}$ decreased in drought-stressed plants by ~43% and 37% during one day recovery after rewatering, respectively (Fig. 2A), which is consistent with previous results in *Xanthium strumarium* (Zeevaart 1980) and served to validate the experimental system for ABA determinations, notwithstanding observed higher ABA levels across the board in well-watered recovery treatments.

Unexpectedly, in the ABI5$^{1\text{-}1\text{-}1}$ and RAV1$^{1\text{-}1\text{-}5}$ lines ABA levels trended higher than control (Fig. 2A; significantly for RAV1) after re-watering for drought recovery, suggesting a homeostatic mechanism for ABA sensitivity and ABA metabolism may operate as observed in Arabidopsis (Leung *et al.* 1997; Liu *et al.* 2015). Consistently, in the well-watered control for drought recovery (eight days after initiation of the experiment), all the lines had significantly higher ABA levels than lines harvested the day before, and ABI$^{1\text{-}1\text{-}1}$ had significantly lower ABA level than Coker 312, a trend also observed for RAV1$^{1\text{-}1\text{-}5}$. In ABI5$^{13\text{-}4\text{-}1}$ and ABI5$^{1\text{-}1\text{-}1}$, ABA levels (albeit non-significant for ABI5$^{1\text{-}1\text{-}1}$) in response to drought treatment (Fig. 2A) were inversely correlated with apparent ABI5 protein accumulation in seeds (Fig. 1; slightly more ABI5$^{1\text{-}1\text{-}1}$ than ABI5$^{13\text{-}4\text{-}1}$). RAV1$^{1\text{-}1\text{-}5}$ and ABI5$^{1\text{-}1\text{-}1}$ had a trend towards lower ABA levels in response to drought, 48% ($P < 0.06$) and 28% lower, respectively, than Coker 312 (Fig. 2A), which is consistent with the pattern seen for well-watered plants after eight days (Fig. 2A) and previous molecular and physiological results that showed these transgenics have a "less stressed" phenotype (Mittal *et al.* 2014, 2015). With the caveat that relative AtABI5 protein levels in

seeds (Fig. 1) may not reflect similar levels in leaves, taken together with prior results for phenotypic effects of these specific ABI5 events correlating with increases in leaf area, WUE, root biomass, and fiber length (Mittal *et al*., 2014, 2015), the immunoblot and ABA quantifictation results are consistent with the working hypothesis of increased ABA sensitivity/homeostasis in ABI5- and RAV1 over-expressing lines.

RAV1$^{1\text{-}1\text{-}5}$ ***cotyledonary leaves have smaller guard cell apertures and higher stomatal density during drought stress and recovery***

Guard cell pores (i.e., stomatal apertures) are the primary means of water loss and CO_2 uptake in plants. Recent work has revealed that guard cell ABA sensitivity increases as the leaf ages, and ABA controls plasticity of stomatal patterning in cotyledons (Chater *et al.* 2014; Pantin *et al.* 2013; Serna 2014; Tanaka *et al.* 2013). Drought stress conditions cause guard cell pores to shrink in size (literally area as measured, μm^2) to minimize water loss, and to increase in size during drought recovery to promote uptake of CO_2, thereby enhancing photosynthetic capabilities. Guard cell pores can change in two different ways when observed in two dimensions by microscopic analysis of epidermal casts: (i) the area (μm^2) of the guard cells (i.e., stomata) *per se* could change by fluctuations in plant turgor pressure, with the pore area (μm^2) changing proportionally, or (ii) the area (μm^2) of guard cell pores might change differently with respect to the overall size of the guard cells. To tease apart the two different possibilities for changes in guard cell pores, we quantified the area of both guard cells and their pores, and analyzed the relationship between pore size and guard cells (i.e., pore area / guard cell area).

Coker 312 had the largest guard cell pores under drought stress conditions (Fig. 2B). In response to drought recovery, guard cell pores of Coker 312 increased in size in both absolute (~6%, Fig. 2B) and proportional terms (22%, $P < 0.05$, Fig. 2C). The transgenic line RAV1$^{1\text{-}1\text{-}5}$ had the smallest guard cell pores under drought-stress conditions (26% smaller than control Coker 312, $P < 0.05$, Fig. 2B). Furthermore, drought-stressed RAV1$^{1\text{-}1\text{-}5}$ plants had disproportionally smaller pores (after correcting for the size of guard cells) than Coker 312 (19% smaller, $P < 0.05$, Fig. 2C). In response to drought recovery, guard cell pores increased by ~20% in RAV1$^{1\text{-}1\text{-}5}$ ($P < 0.05$), but remained significantly smaller (~21%) than Coker 312 ($P < 0.05$, Fig. 2B). Similarly, the proportional size of RAV1$^{1\text{-}1\text{-}5}$ guard cell pores increased by 11% during drought recovery ($P < 0.001$), but remained significantly smaller (26%) than Coker 312

($P < 0.05$, Fig. 2C). For ABI5$^{1\text{-}1\text{-}1}$, the absolute (Fig. 2B) and proportional sizes (Fig. 2C) of guard cell pores trended smaller than Coker 312 under drought stress. The proportional size of ABI5$^{1\text{-}1\text{-}1}$ guard cells pores (but not absolute size) during drought recovery was significantly smaller than Coker 312 (Fig. 2C, $P < 0.05$). For the ABI5$^{13\text{-}4\text{-}1}$ line, the absolute and proportional sizes of guard cell pores trended smaller than Coker 312 under drought stress. Drought recovery in ABI5$^{13\text{-}4\text{-}1}$ caused a ~40% and 35% increase ($P < 0.05$) in the absolute and proportional sizes of guard cell pores, respectively. The absolute size of guard cell pores trended larger than Coker 312 during drought recovery, while the proportional size of guard cell pores was significantly smaller ($P < 0.05$). These results suggest that RAV1 and ABI5 transgenics may have reduced ABA sensitivity and/or ABA metabolism that impact stomatal development and function, as has been observed in Arabidopsis.

Since our prior work with these representative and other independent RAV1 transgenic cotton lines found transgene effects on photosynthesis and stress response physiology, we measured stomatal density in cotyledons. Figure 3 shows that the RAV1$^{1\text{-}1\text{-}5}$ overexpression line had significantly more stomata on the abaxial side of cotyledons than Coker312, and that ABI5$^{1\text{-}1\text{-}1}$ genotype trended toward higher stomatal density, correlating with observed significantly lower ABA levels (Fig. 2A) and work of Tanaka *et al.* (2013) who showed the ABA-deficient *aba2-2* mutant of Arabidopsis has increased number/proportion of stomata and reduced expansion of cotyledon pavement cells mediated by SPCH and MUTE, master regulators for stomatal formation. The observed trend for ABI5$^{13\text{-}4\text{-}1}$ stomatal density was slightly lower (Fig. 3) than Coker312 but not statistically significant, and was in line with ABA concentrations and trend of lower guard cell relative pore areas compared to Coker312 (Fig 2A,C), consistent with prior results showing ABI5$^{13\text{-}4\text{-}1}$ had generally lower WUE than ABI5$^{1\text{-}1\text{-}1}$ in the field (Mittal *et al*. 2014) and was not considered further.

Lower cotyledon surface temperatures in drought-stressed transgenic plants

We measured LST to infer transpiration stress physiology of the ABI5 and RAV1 transgenic lines. Our prediction was that drought-stressed plants would have higher LSTs than well-watered plants due to lower transpiration rates under drought-stressed conditions (Grant *et al.* 2006). The results for LSTs are shown in Fig. 4. In general, our results for all the cotton plants supported the prediction of higher LST for drought-stressed plants. Coker 312 plants

showed a trend of cooler LSTs as the experiment progressed, possibly due to developmental increases in ABA sensitivity/physiological homeostatic mechanisms as observed in Arabidopsis (Leung et al. 1997; Liu et al. 2015). Coker 312 plants under drought stress had significantly higher LST than well-watered plants ($P < 0.01$). As hypothesized and consistent with ABA dynamics (Fig. 2A), there was a marked reduction in LST during drought recovery, especially for RAV1$^{1\text{-}1\text{-}5}$ and ABI5$^{1\text{-}1\text{-}1}$ lines (Fig. 4B,C).

Under peak drought conditions (7D-Drt), all three transgenic cotton lines had lower LST than Coker 312, with ABI $^{1\text{-}1\text{-}1}$ having the lowest LST ($P < 0.05$; Fig. 4E), consistent with observed ABA dynamics (Fig. 2A). In addition, the relative increase in LST as the drought experiment progressed was highest for Coker 312. On days five through seven of the drought treatment, Coker 312 averaged a daily increase in LST of 1.7^{o} C, whereas RAV1$^{1\text{-}1\text{-}5}$, ABI5$^{1\text{-}1\text{-}1}$, and ABI5$^{13\text{-}4\text{-}1}$ averaged 1.1, 0.4, and 0.4^{o} C increases per day, respectively (Fig. 4A-D). The lower observed LST of transgenics versus Coker312 during drought is contradicted by observed smaller guard cell pore areas of transgenics (Fig. 2C), but can be reconciled by the higher observed stomatal densities in the transgenics (Fig. 3). Together, these results allow inference that the transgenics had higher transpiration rates, manifest as lower LST, under drought stress conditions and had more stable transpiration rates over the course of the drought stress period.

RAV1$^{1\text{-}1\text{-}5}$ ***and*** **ABI5$^{1\text{-}1\text{-}1}$** ***mature leaves have higher stomatal conductance and photosynthetic rates under drought stress***

We previously characterized the molecular and physiological phenotypes of four independent transgenic events for AtRAV1 and the two AtABI5 events studied here and showed that AtRAV1$^{1\text{-}1\text{-}5}$ is expressed lower than AtRAV1$^{13\text{-}7\text{-}2}$ in leaves and has less extreme phenotypes than AtRAV1$^{13\text{-}7\text{-}2}$ of longer fiber length, larger leaf area, longer internode length, and increased WUE under well-watered and drought conditions in the greenhouse and field (Mittal *et al.*, 2014, 2015). Those studies likewise established the AtABI5$^{1\text{-}1\text{-}1}$ event has more extreme phenotypic effects than AtABI5$^{13\text{-}4\text{-}1}$ for longer fiber lengths, larger leaf area, greater root biomass, and higher WUE. To integrate the working hypothesis from cotyledonary to mature leaves, we directly measured photosynthetic parameters including stomatal conductance in five- week-old greenhouse-grown plants, and the results are shown in Fig. 5. Coker 312 (wild-type) plants had a strong wilting phenotype in the afternoon of day four after withholding

water (data not shown). This strong drought stress resulted in significant and progressive inhibitory effects on stomatal conductance and photosynthesis, which were partially relieved after two days of re-watering. The ABI5$^{1\text{-}1\text{-}1}$, ABI5$^{13\text{-}4\text{-}1}$, RAV1$^{1\text{-}1\text{-}5}$, and RAV1$^{13\text{-}7\text{-}2}$ transgenic lines all showed significantly higher stomatal conductance at six days of drought (Fig. 5), where the greater number of biological replicates allowed statistical inference, and under drought stress did not show wilting symptoms (data not shown), as previously shown (Mittal *et al.* 2014). The RAV1$^{13\text{-}7\text{-}2}$ and ABI5$^{1\text{-}1\text{-}1}$ transgenic lines at six days of drought showed significantly higher photosynthetic rates, whereas ABI5$^{13\text{-}4\text{-}1}$ and RAV1$^{1\text{-}1\text{-}1}$ photosynthetic rates trended 19- 27%, higher respectively, than Coker 312 consistent with prior results (Mittal *et al*. 2014, 2015) and data presented in Figs. 1, 2, and 4.

***GhDREB*, an effector of ABA response, is up-regulated in RAV1 under well-watered conditions and down regulated in RAV1$^{13\text{-}7\text{-}2}$ and ABI5$^{13\text{-}4\text{-}1}$ transgenics under drought and recovery treatments**

In order to characterize RAV1 and ABI5 transgenic cotton at the level of transgene activity, we quantified endogenous transcript abundance of a *DREB/CBF* (dehydration-responsive element/C repeat binding factor) in a greenhouse drought stress and recovery experiment. DREB TFs specifically bind to dehydration-responsive element (DRE)/C-repeat (CRT) cis-acting promoter elements (A/GCCGAC) and are sufficient for transactivation of many stress-inducible genes (Maruyama *et al*. 2004). We examined *GhDREB* expression in the transgenics as a molecular marker for stress signaling state.

Fig. 6 shows an RNA blot result for *GhDREB* transcript in well-watered, drought- and recovery stage in transgenic cotton lines. *GhDREB* was highly induced in response to drought (~5.7 fold ; compare lanes 4 and 8 "Drt/WW") . In response to re-watering its expression was reduced (to ~0.3 fold of the drought expression; compare lane 8 and 12 "Rec/Drt"). Its expression was higher in unstressed RAV1 and a RAV1 x ABI5 stacked line compared to control (1.34-1.27 fold, respectively; compare lane 1 and 3 to lane 4). Remarkably, its expression was lower in all the transgenics and RAV1 x ABI5 cross (0.42-0.60 fold reduction; compare lanes 5-7 to lane 8; "Line/Coker in specified treatment") compared to wild type Coker in response to imposed drought stress. Interestingly, even during the recovery stage its expression was 0.50-0.65 fold (lower) in the transgenics and stacked cross compared to wild type. Taken together

with prior results that stress-responsive DREBs activate expression of stress-inducible genes and improve tolerance to not only drought, salinity, and freezing but also growth retardation (Maruyama et al. 2004), our result indicates that RAV1, and to a lesser extent ABI5, unstressed and drought-stressed transgenic plants exhibit a "less stressed phenotype". In order to further substantiate that constitutive overexpression is beneficial for transgenics plants in terms of overall growth performance, we measured root and shoot biomass in 90 DAP greenhouse-grown lines under well-watered and drought stress conditions. Results shown in Supplemental Fig. S1 extend prior results from field and greenhouse (Mittal *et al*. 2014, 2015) that RAV1 overexpressing- and ABI5$^{1\text{-}1\text{-}1}$ greenhouse-grown lines have significantly increased root biomass compared to control Coker312 under well-watered and drought-stressed conditions, which trended to increased shoot biomass under well-watered conditions in RAV1 and ABI5 x RAV1-stacked lines.

Discussion

Among the three transgenic lines tested, RAV1$^{1\text{-}1\text{-}5}$ had the lowest ABA levels during drought stress compared to control Coker312. The evidence showing ABI5$^{1\text{-}1\text{-}1}$, ABI5$^{13\text{-}4\text{-}1}$, RAV1$^{1\text{-}1\text{-}5}$, and RAV1$^{13\text{-}7\text{-}2}$ plants had significantly higher stomatal conductances and higher photosynthesis rates (Fig. 5) during extreme drought stress, and corresponding lower cotyledonary LSTs (as a proxy for higher whole leaf transpiration rates)(Fig. 4) support a "less stressed" phenotype under drought stress, which agrees with previous results (Mittal *et al.* 2014, 2015). We frame a model of sensitivity to ABA in transgenics affecting stomatal development/physiology that integrates results of lower ABA levels during drought and higher ABA levels during recovery (Fig. 2A) which correlate with relative guard cell pore areas (Fig. 2C), including during drought recovery when LSTs completely recover to unstressed levels for ABI5^1-1-1 and RAV1^1-1-5 (Fig. 4). The results of lower ABA contents and smaller guard cell pore areas for the transgenic lines AtRAV1$^{1\text{-}1\text{-}5}$ and AtABI5$^{1\text{-}1\text{-}1}$ under unstressed or drought conditions (Fig. 2), while at the same time having higher stomatal densities, stomatal conductance resulting in leaf evaporative cooling (lower LST), and photosynthetic rates than wild type counterparts (Figs. 3-5) are reconciled by inferring an increased ABA sensitivity in the transgenics (less ABA improves physiology generally, i.e. "less stressed" ~ higher function). This interpretation is consistent with the observation that during recovery from drought stress, ABA levels of these two transgenic lines 'flipped' and became elevated compared to wild type

control (Fig. 2A), suggesting changes in temporal fluxes of ABA during and after stress. It is postulated that three major processes are involved in the over-expression lines: altered regulation of ABA biosynthesis/catabolism (Liu *et al.* 2015; Zeevaart 1980), altered ABA sensitivities *per se*, and activity of a known ABA homeostatic feedback loop (Leung *et al.* 1997). Further studies using $^{18}O_2$ to quantify metabolic fluxes to and from ABA in the transgenics could shed light on the issue of ABA metabolic fluxes in the transgenics, as has been shown for an Arabidopsis mutant of ABA signaling (Xiong *et al*. 2001).

A plausible mechanism of the observed RAV1 guard cell phenotypes is through repression of endogenous FT, as has been shown in Arabidopsis (Ando *et al.* 2013; Castillejo and Pelaz, 2008; Kinoshita *et al.* 2011) and in RAV1 and RAV2-overexpressing cotton lines (Mittal *et al.* 2015). Although these results were obtained with embryonic leaves and therefore extrapolation to later stages of development is qualified due to possible differences in tissue specificity/sensitivity, we interpret these results as representative of older leaf tissue because: i) we observed complimentary results for mature leaf stomatal conductance (Fig. 5A) as we did for whole cotyledon transpiration rates measured as LST (Fig. 4); ii) we previously showed in Arabidopsis that the *abi1* and *abi2* mutants, which are impaired in ABA sensitivity by disruption of ABA receptor activity (Cutler *et al.* 2011), show similar effects on guard cell-specific ABA-inducible gene expression in cotyledonary leaves (Chak *et al.* 2000); and iii) recent work on leaves of various developmental stages corroborates this interpretation that guard cells of cotyledons and true leaves respond to ABA similarly, albeit through homeostatic interplay of stomatal patterning plasticity and ABA sensitivity mediated by downstream signaling components and enlargement of pavement cells in cotyledons (Chater *et al.* 2014; Pantin *et al.* 2013; Serna 2014; Tanaka *et al.* 2013).

Our initial hypothesis was the transgenic lines would exhibit drought tolerance through an enhanced responsiveness to ABA, because previous research has shown that these transgenes are effectors of (i.e., downstream respondents to endogenous) ABA. Somewhat unexpectedly, despite smaller pore spaces and stomatal opening, the cotyledons of independent AtRAV1 and AtABI5 events had increased stomatal conductance (Fig. 5) and transpirational cooling (Fig. 4, observed LST as proxy for transpiration rate). The finding that stomatal densities in the ABI5$^{1\text{-}1\text{-}1}$ and RAV1$^{1\text{-}1\text{-}5}$ transgenic cotyledonary leaves (Fig. 3) correlated with ABA accumulation dynamics (Fig. 2A) and lower LST (Fig. 4) during and after drought stress supports the model,

with the caveat we did not measure stomatal developmental dynamics during the timecourse of the experiments. ABA directly affects guard cell physiology (Kim *et al.* 2010), and in our study guard cell pores of RAV1$^{1\text{-}1\text{-}5}$ were 26% smaller during drought stress than control Coker312. Interestingly, after correcting for the size of the guard cells, the guard cell pores of drought-stressed RAV1$^{1\text{-}1\text{-}5}$ plants were disproportionally smaller (19%) than control Coker312, a trend that held for both ABI5 transgenics (Fig. 2C). Given that lower levels of ABA in RAV1$^{1\text{-}1\text{-}5}$ correlated with greater stomatal density and a larger reduction in guard cell pores than in control Coker312, which in turn correlated with lower LST (Fig. 4) and higher stomatal conductance (Fig. 5), taken together we interpret these data as consistent with a hypersensitive response of transgenic guard cell pores to ABA, concomitant with other known attendant drought adaptation mechanisms, e.g. feedback homeostasis of ABA sensitivity (Cutler *et al.* 2011; Leung *et al.* 1997) and catabolism (Liu *et al.* 2015). Hypersensitivity to ABA through over-expression of RAV1 was also found by Sohn *et al.* (2006) who showed that exogenous ABA applied to Arabidopsis lines over-expressing a heterologous RAV1 resulted in ABA-hypersensitive inhibition of germination and root elongation. Interestingly, in that study, over-expression of capsicum RAV1 also resulted in enhanced resistance to the pathogen *Pseudomonas syringae* pv. Tomato DC3000, which infects hosts through guard cell pores (Xin and He 2013). This result is intriguing in the context of our data showing smaller guard cell pores in over-expressing AtRAV1 cotton plants, and begs the question of whether smaller guard cell pores in Arabidopsis lines over-expressing RAV1 explain their enhanced resistance to pathogen infection. Moreover, guard cell pores serve many critical functions in plants (i.e., water loss, CO_2 uptake, and pathogen susceptibility) and, in light of our results and those by Sohn *et al.* (2006), RAV1 transgenics may have value for the development of drought-tolerant and pathogen-resistant crop varieties. RAV1 has recently been shown in Arabidopsis to be genetically upstream of ABI5 in ABA regulation of seed germination and subject to ABA-dependent SNF1-RELATED PROTEIN KINASE SnRK2 negative regulation by phosphorylation (Feng *et al*., 2014). We speculate that the low levels of c-myc::RAV1$^{1\text{-}1\text{-}5}$ observed in transgenic seeds (Fig. 1) may be due to homeostatic feedback of ABA responses (Leung *et al.* 1997), or possibly targeted degradation by interaction with the response regulator GIGANTEA (Sawa and Kay, 2011), a general protein chaperone with myriad roles in growth, development, and stress responses (Cha *et al*. 2017) and itself subject to ABA modulation (Riboni *et al*. 2016). Such feedback loops

resulting in low AtRAV1 in seeds (assumed low in cotyledons) complicates interpretation of observed phenotypic effects of "overexpression." Our demonstration that drought-inducible expression of an endogenous positive effector of ABA responses, *GhDREB*, as molecular marker of ABA sensitivity correlates with AtABI5- and AtRAV1 overexpression phenotypes (Mittal *et al*. 2014, 2015) and physiology (Figs. 2-5) under well-watered and drought-stress/response treatments (Fig. 6) establishes the transgene products are functional and can interact. Our results suggesting increased stomatal sensitivity to ABA and those of Sohn *et al*. (2006) appear at odds with those of Feng *et al*. (2014) who showed lowering endogenous *RAV1* in Arabidopsis results in ABA hypersensitivity for inhibition of seed germination. Further work is required to elucidate the molecular mechanisms of AtRAV1 action in different tissues of transgenic cotton.

The "less stressed" phenotypes observed for AtRAV1$^{1\text{-}1\text{-}5}$ and AtRAV1$^{13\text{-}4\text{-}1}$ could be due to better WUE through disproportionally smaller guard cell pores. Mittal *et al.* (2014) found an additional mechanism of drought avoidance in these AtRAV1and AtABI5 events by showing that drought-stressed plants grew ~12-80% more root dry biomass in the greenhouse than Coker 312. We have also observed a late flowering phenotype of these AtRAV1 and AtRAV2 over-expressing cotton lines grown in the field (Mittal *et al.* 2015), supporting the drought avoidance mechanism. Taken together, the "less stressed" phenotype by RAV1 over-expression may therefore be due to multiple mechanisms of drought avoidance (late flowering, greater root biomass) and drought tolerance (improved WUE through stomatal regulation and attendant increases in photosynthesis) (Fig. 5).

We found evidence of a drought tolerance phenotype in ABI5$^{1\text{-}1\text{-}1}$ based on lower ABA levels, lower LST, and higher stomatal conductance and photosynthetic rates than control Coker312. However, we were unable to strictly correlate this phenotype with another transgenic event ABI5$^{13\text{-}4\text{-}1}$ based on apparent ABI5 protein expression (in seeds, Fig. 1) or changes in stomatal density (Fig. 3) or guard cell pore sizes (Fig. 2B~~,C~~), which suggests there may exist alternative mechanisms for drought tolerance/avoidance in ABI5$^{1\text{-}1\text{-}1}$ than postulated for AtRAV1 in this study. However, Mittal *et al.* (2014, 2015) provided evidence of drought-avoidance traits in both ABI5$^{13\text{-}4\text{-}1}$ and ABI5$^{1\text{-}1\text{-}1}$ events, namely longer fiber lengths, larger leaf area, greater root biomass, and higher WUE, the degrees of which correlated with phenotypic effects of these lines characterized in this study. Furthermore, stacked transgenic lines from crosses between ABI5 and AtRAV1 (Fig. 6) and AtRAV2 showed synergy in plant yield, among other responses

(Mittal *et al.* 2014). It seems possible therefore that multiple mechanisms of drought tolerance and avoidance operate in ABI5 and RAV1 lines studied here, which could potentially explain why stacked transgenic lines of ABI5 plus RAVs showed synergies in some, but not all, characterized phenotypes (Mittal *et al.* 2014, 2015).

The results of this study suggest that over-expression of RAV1 results in drought tolerance through adaptive hypersensitive responses of guard cells to endogenous ABA. Given that Mittal *et al.* (2014, 2015) also found drought avoidance in RAV1 and AtABI5 overexpression lines through greater root dry biomass, it is possible that over-expression of RAV1 or ABI5 influences multiple plant traits which each contribute to different facets of a complex of drought adaptation phenotypes.

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Conflict of Interest: Two of the authors (CDR, AM) have filed (June 9, 2017) a USPTO patent application #62089567 entitled "Transcription factors and method for increased fiber length of cotton." The authors declare that they have no conflict of interest. The sponsors had no role in the design and conduct of the study; in the collection, analysis, and interpretation of data; or in the preparation, review, or approval of the manuscript or decision to submit the article for publication.

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Figure Captions and Legends

Fig. 1 Relative quantification of transgene products and mRNAs in seeds of independent events and transient gene expression assays. A) Immunoblot of proteins extracted from seeds of transgenic AtABI5 and AtRAV1 overexpressing lines and from leaves of *Nicotiana benthamiana* ("bBenth") transiently transformed with Agrobacterium harboring the same T-DNA vector (Guo and Ecker, 2003; Mittal *et al*. 2014) for production of c-myc N-terminal epitope tagged-RAV1 and -ABI5 stable cotton transformants. ~~A)~~ Upper panel; iImmunoblot probed with monoclonal anti c-myc antibody. c-myc:ABI5 protein expected size is 61 kDa; c-myc:RAV1 protein expected size is 52 kDa (inset arrow). Middle panel:~~B) Same blot probed with~~ immunoblot probed with anti-ubiquitin antibody~~,~~ as loading control; MW ~8.5 kDa. Below is relative c-myc quantification (± s.e.m., n= 5) by band dosimetry, normalized to ubiquitin. Lower panel:~~C)~~ Coomassie-stained gel, marking migration of Rubisco large subunit, MW ~56 kDa. B) RNA blot from *N. benthamiana* leaves transformed with Agrobacterium harboring the same T-DNA vectors for production of tagged-RAV1 and -ABI5 stable cotton transformants, probed with *LEA-5* marker 24 hours after innoculation. C) RNA blot probed with *ERD10C* from *N. benthamiana* transient assay showing response after one hour treatment with 50 µM ABA. D) Average relative dosimetric quantification of RAV1 and ABI5 overexpression effects on ABA response sensitivity of markers *LEA-5* and *ERD10C* in *N. benthamiana* transient assays measured 24 hours after inoculation, compared to ABA response after one hour.

Fig. 2 Changes in the (A) abscisic acid and (B,C) guard cell pore metrics in response to 7 days drought-stress (7D-Drt) and 1 day recovery (1D-Rec) in cotyledonary leaves of transgenic cotton (*Gossypium hirsutum* L.) over-expressing AtABI5 or AtRAV1. WW, well watered; DS, drought

stressed. For panel B, each data point represents the summed area of 15 guard cell pores from an individual cotyledonary leaf. For panel C, the area of guard cell pores was divided by the area of the guard cells (stomata). Analysis of plant hormones was conducted using mass spectrometry (Supplemental Table S1). Bars represent means ± S.E.; n = 3 per treatment. Asterisk (*) indicates significantly different ($P < 0.05$) than Coker312; plus (+) indicates significant difference between well-watered (WW) and drought-stressed (DS) plants for a specific cotton line; caret (^) indicates significant effect of drought recovery treatment for a specific cotton line.

Fig. 3 Stomatal densities on abaxial side of 13-day-old cotyledons of Coker 312 and AtABI5 and AtRAV1 overexpressing transgenics. Bars represent mean density ± S.E.; n= 11-12 individual plants per genotype, seven to eight images per plant. Asterisk (*) indicates significantly different ($P < 0.00001$) from Coker312 control.

Fig. 4 Timecourse of drought effects and recovery one day after watering (1D-Rec) on cotyledonary leaf surface temperature in transgenic cotton (*Gossypium hirsutum* L.) lines over-expressing AtABI5 or AtRAV1. A) Control Coker312. B) AtRAV1$^{1\text{-}1\text{-}5}$. C) AtABI5$^{1\text{-}1\text{-}1}$. D) AtABI5$^{13\text{-}4\text{-}1}$. E) Histogram for genotype effects on leaf surface temperature after seven days drought (7D-Drt). Well-watered (solid lines) plants were given water on days 4 and 7 after initiation of drought stress (solid arrow; 4D-Drt and 7D-Drt), whereas drought-stressed plants (dashed lines) were given water on day 7 only (dashed arrow). Bars represent means ± S.E.; n = 10-12 on days 4-7D, n = 7-9 on day 1D-Rec. Asterisk (*) indicates significantly different ($P < 0.05$) than Coker312.

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Supplemental Fig. S1. RAV1 overexpressing- and ABI5$^{1\text{-}1\text{-}1}$ greenhouse-grown lines (90 DAP) have significantly increased root biomass under well-watered and drought-stressed conditions

compared to control Coker312, which trends to increased shoot biomass under well-watered conditions in RAV1 and ABI5 x RAV1-stacked lines. Error bars are s.e.m., n=10 plants for Coker312, n=3-4 plants for transgenics. Asterisk (*) indicates $P < 0.05$; carat (^) indicates $P < 0.08$ (Student's two-sided t-test, equal variance assumed).

Table S1. Optimized compound-dependent mass spectrometry parameters[a] for hormone[b] quantifictations.

Compound	Q1	Q3	DP	EP	CE	CXP
SA	137	93	-49	-22	-5	-5
ABA	263.1	153	-60	-16.7	-9	-9
JA	209	59	-60	-24	-2	-2
H_2JA	211	59	-60	-24	-2	-2
D_4SA	142	98	-49	-22	-7	-7
OPDA	291.1	165	-75	-30	-5	-5
JA-Ile	322.1	130	-65	-32	-7	-7
D_6ABA	269.1	159	-70	-16	-13	-13

[a]deuterium-labeled (D) D_6ABA was used as the internal standard for ABA, D_4SA for SA, dihydrojasmonic acid (H_2JA) for JA, JA-Ile, and OPDA. Abbreviations of the compound-dependent parameters are as follows: Q1, selected m/z of the first quadruple; Q3, selected m/Z of the third quadruple; DT, dwell time monitoring each MRM transition (ms); DP, de-clustering potential of TIS source (Volts); CE, collision energy (arbitrary unit); CXP, collision cell exit potential (Volts); EP, collision cell exit potential (Volts).

[b]SA: salicylic acid; ABA: abscisic acid; JA: jasmonic acid; JA-Ile: JA-Isoleucine conjugate; OPDA: JA precursor 12-oxo-phytodienoic acid.

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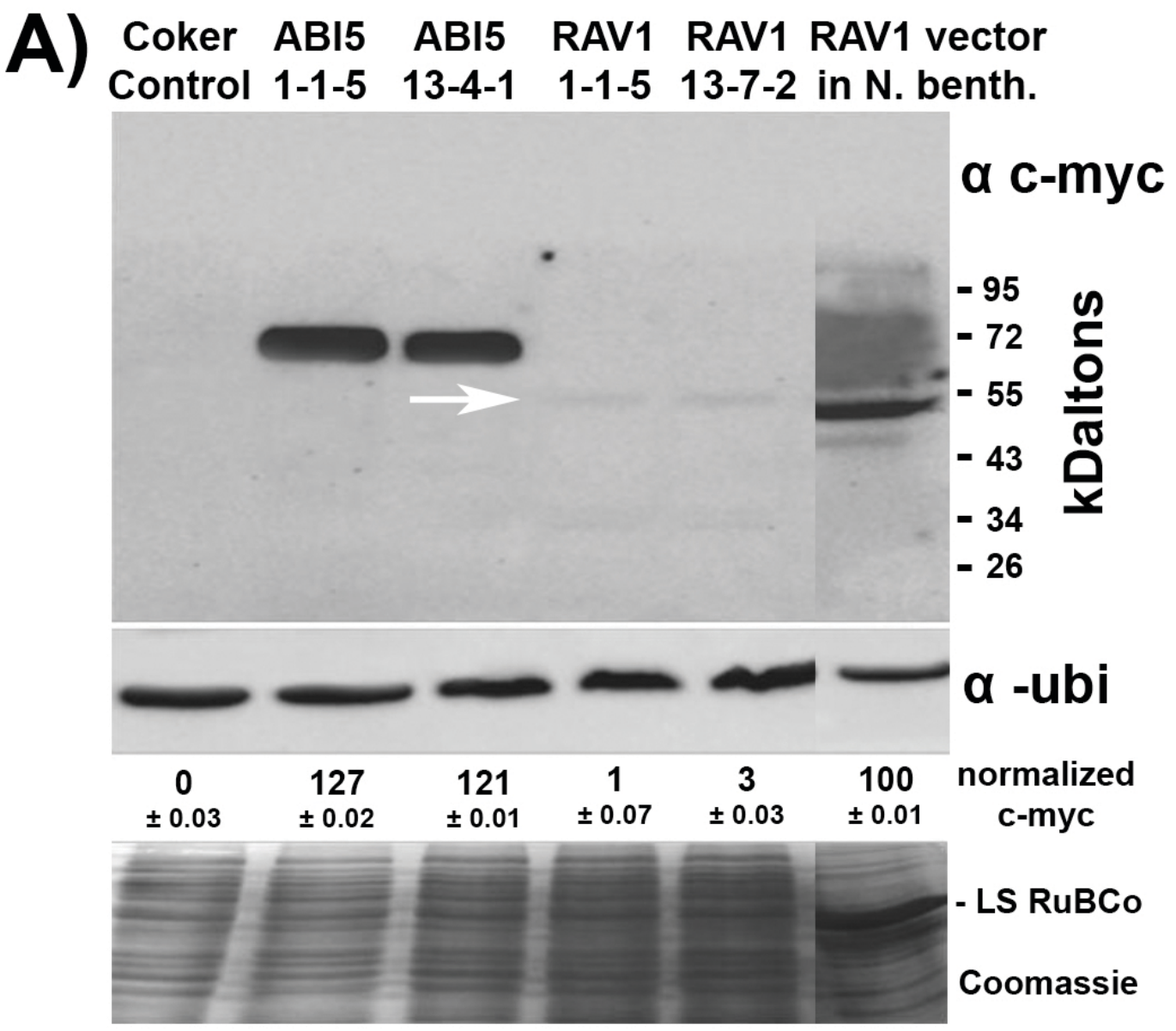

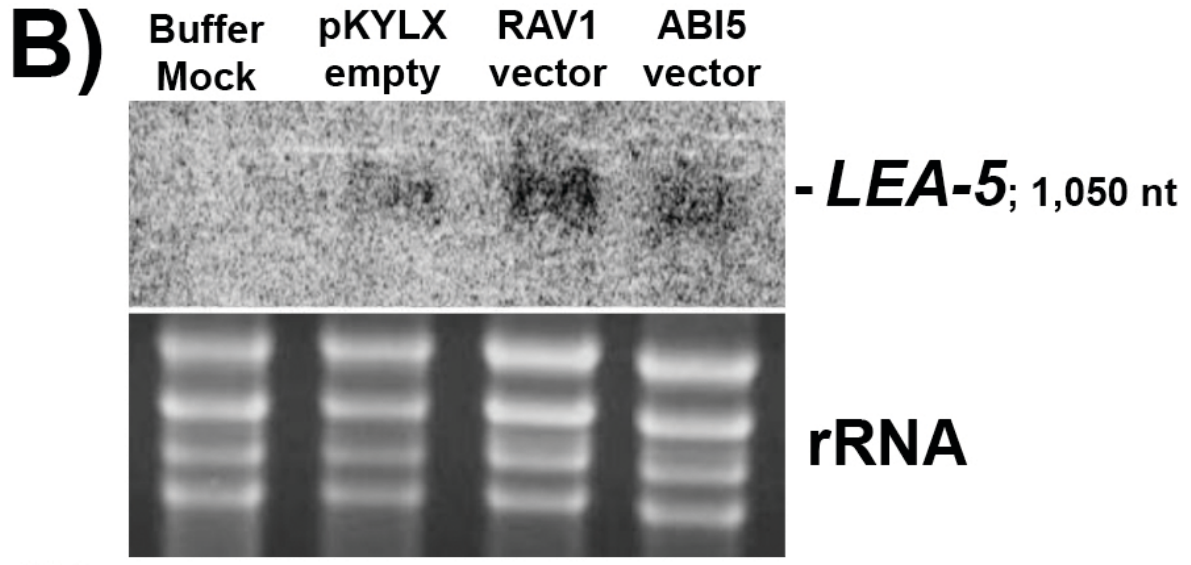

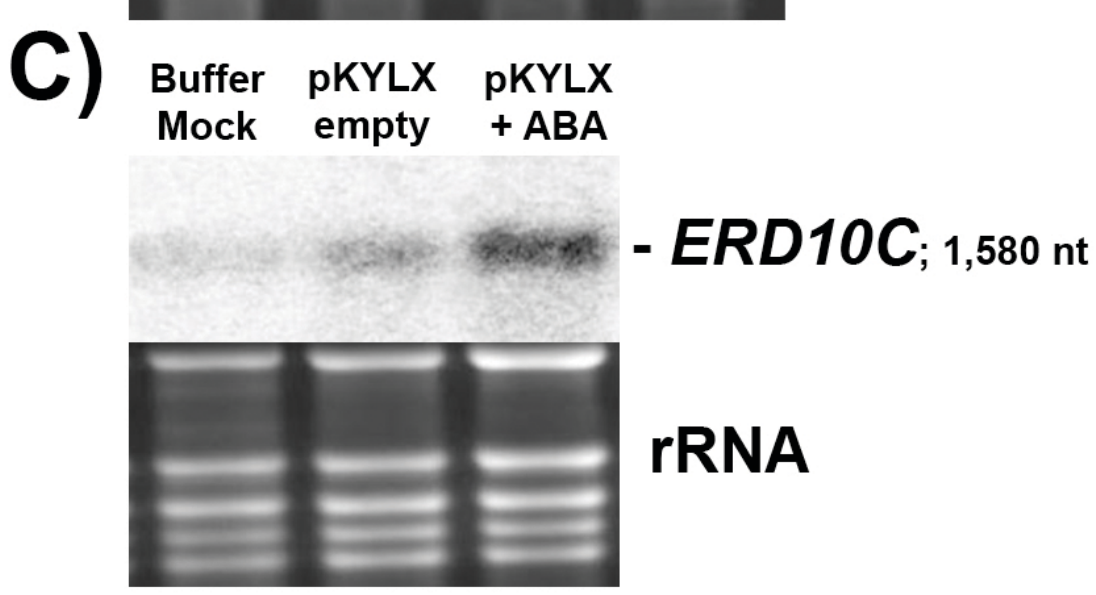

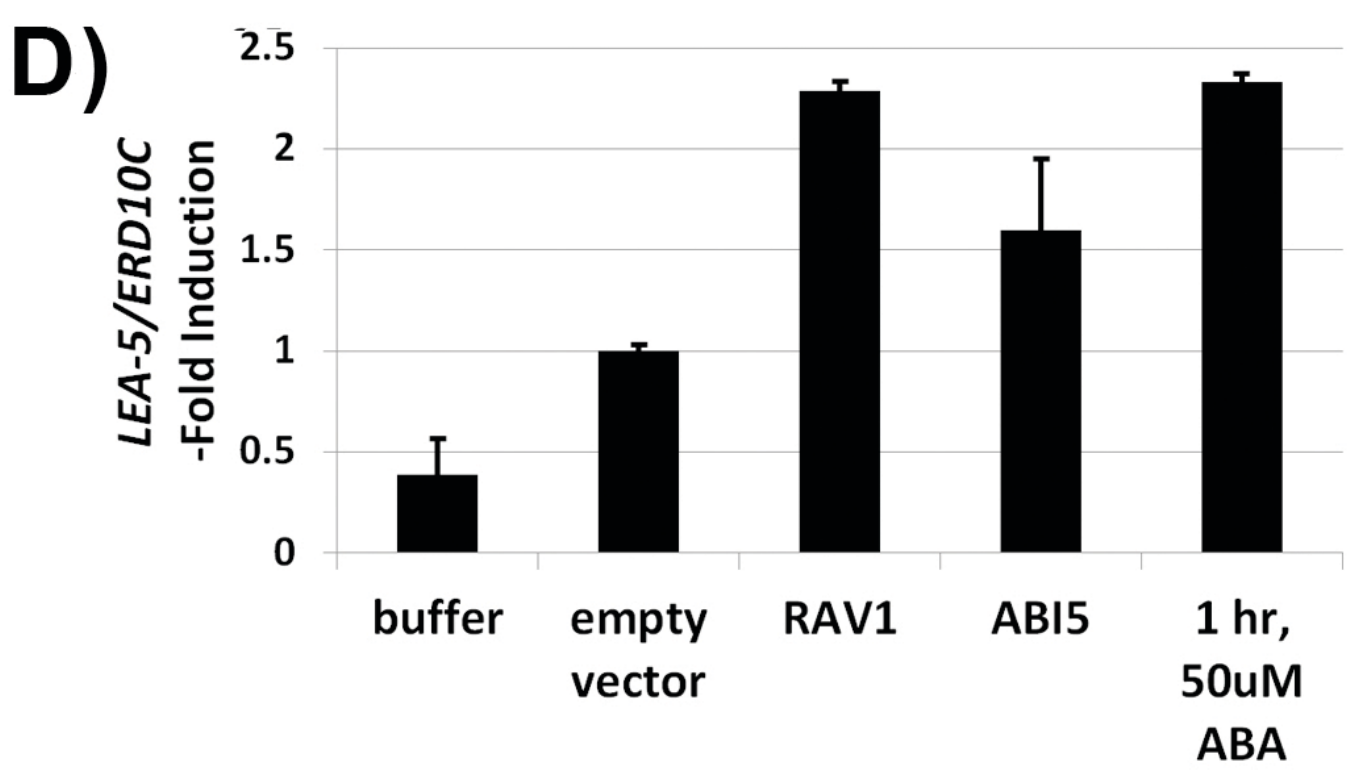

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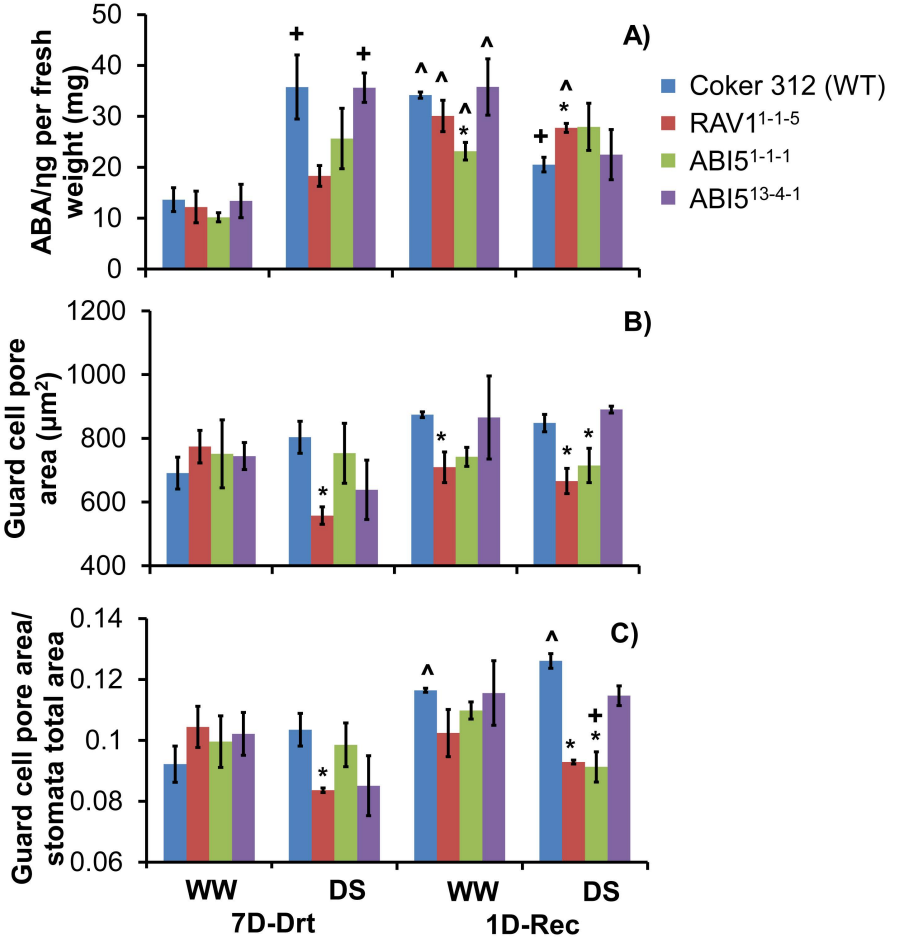

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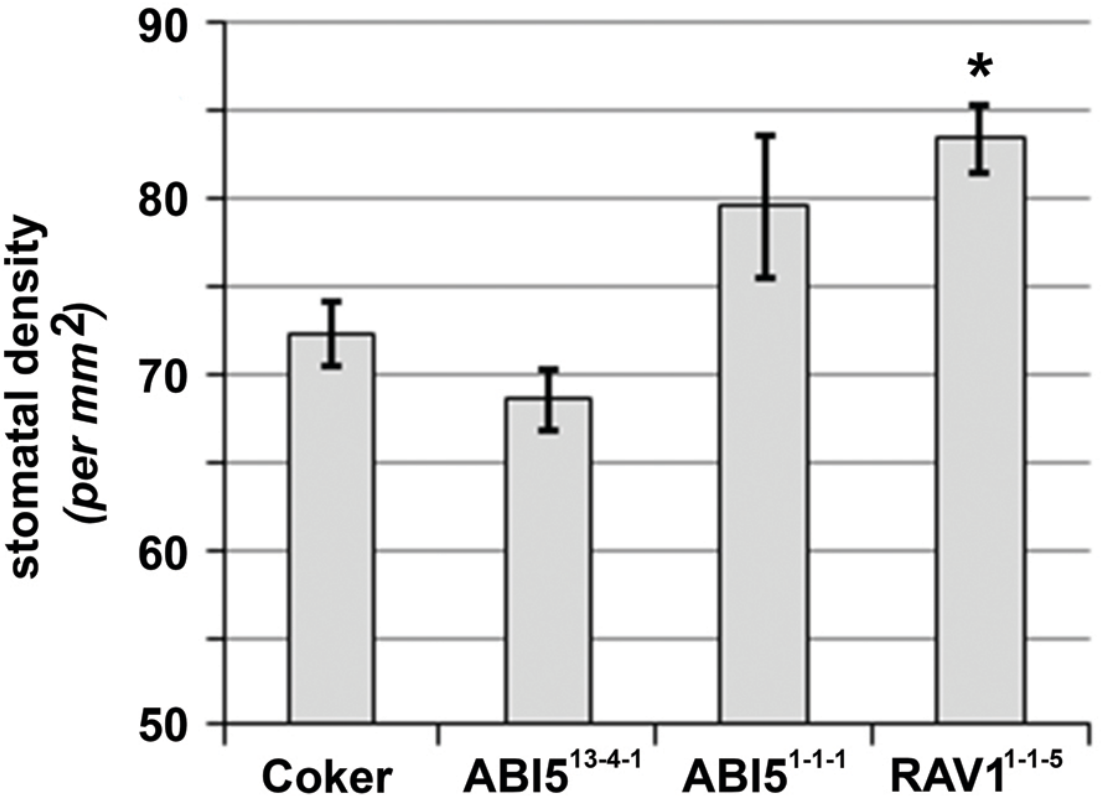

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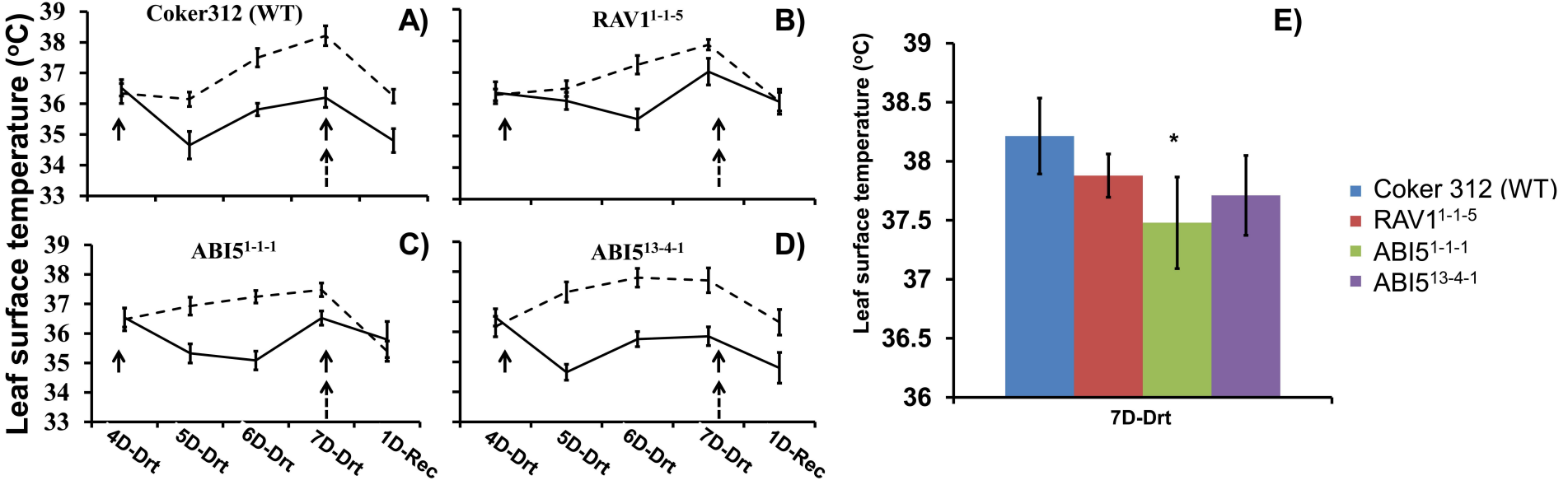

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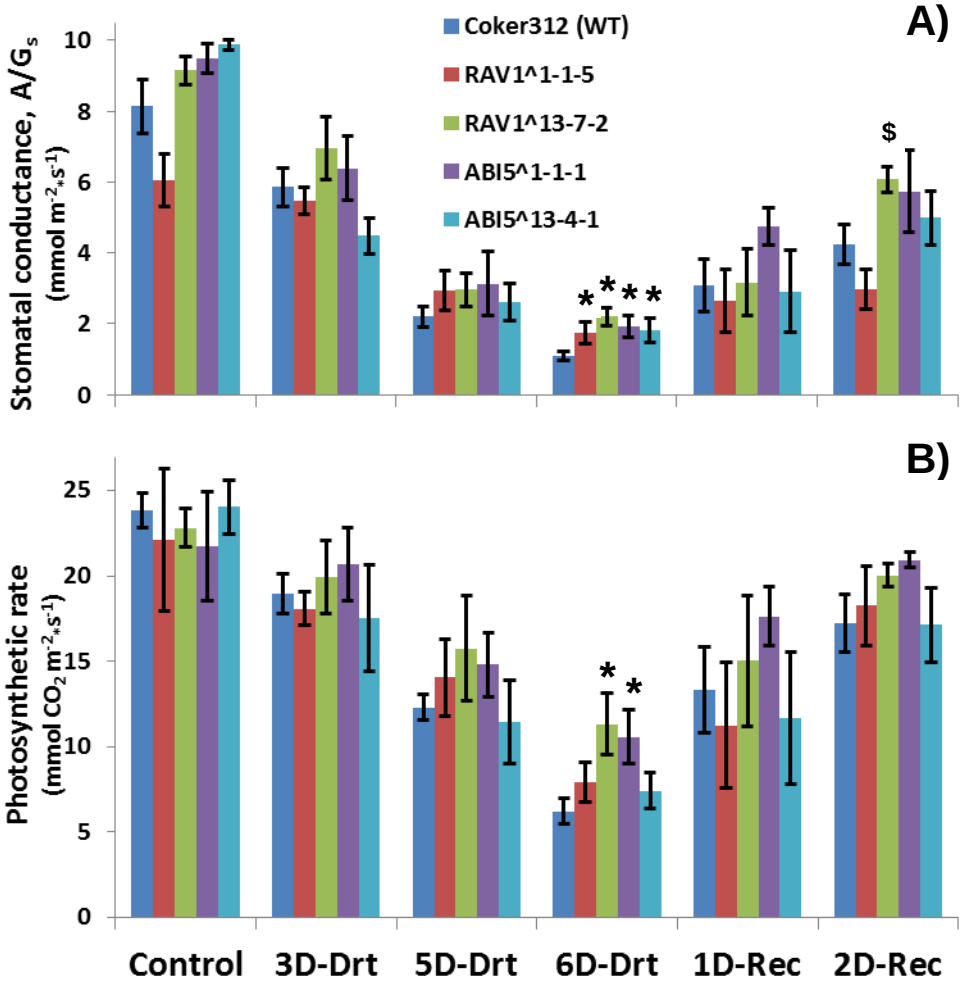

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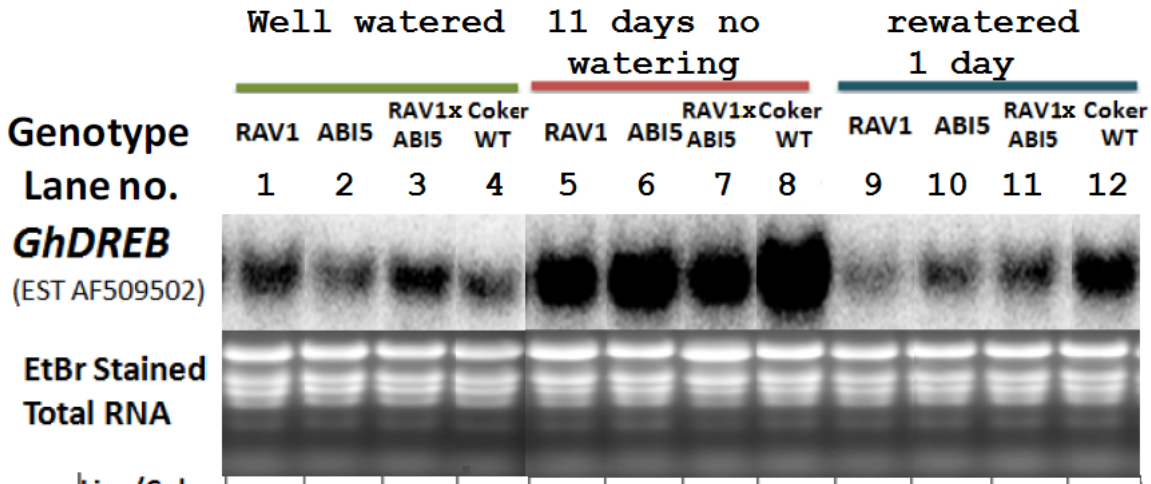

Signal ratio												
	Line/Coker in specified treatment	1.34	1.00	1.27	1	0.44	0.60	0.42	1	0.50	0.57	0.65	1
	Drt/WW					1.88	3.46	1.93	5.77				
	Rec/Drt									0.37	0.30	0.49	0.32
	Rec/WW									0.69	1.05	0.95	1.84

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