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Overexpression of the *Arabidopsis* 14-3-3 Protein GF14λ in Cotton Leads to a "Stay-Green" Phenotype and Improves Stress Tolerance under Moderate Drought Conditions

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The *Arabidopsis* gene *GF14λ* that encodes a 14-3-3 protein was introduced into cotton plants to explore the physiological roles that GF14λ might play in plants. The expression level of *GF14λ* under the control of the cauliflower mosaic virus 35S promoter varied in transgenic cotton plants, and lines that expressed *GF14λ* demonstrated a "stay-green" phenotype and improved water-stress tolerance. These lines wilted less and maintained higher photosynthesis than segregated non-transgenic control plants under water-deficit conditions. Stomatal conductance appears to be the major factor for the observed higher photosynthetic rates under water-deficit conditions. The stomatal aperture of transgenic plants might be regulated by GF14λ through some transporters such as H^+-ATPase whose activities are controlled by their interaction with 14-3-3 proteins. However, since 14-3-3 proteins interact with numerous proteins in plant cells, many metabolic processes could be affected by the *GF14λ* overexpression. Whatever the mechanisms, the traits observed in the *GF14λ*-expressing cotton plants are beneficial to crops under certain water-deficit conditions.

Keywords: Drought tolerance — Leaf senescence — 14-3-3 protein — Photosynthesis — Stomatal conductance.

Introduction

The 14-3-3 proteins are a group of highly conserved regulatory proteins found in eukaryotic cells (Aitken et al. 1992, Ferl 1996). They function as homo- or hetero-dimers and each monomer can bind to an interacting protein (Liu et al. 1995, Xiao et al. 1995, Obsil et al. 2001). There are over one hundred proteins identified as their interacting proteins (Chung et al. 1999, Finnie et al. 1999), and the binding of 14-3-3 proteins to their interacting proteins may change the activities or subcellular localization of those proteins, or mediate formation of protein complexes (van Hemert et al. 2001). In plants, 14-3-3 proteins were shown to regulate primary metabolism, ion transport, cellular trafficking, enzyme activities and gene expression (Ferl 1996, Aducci et al. 2002, Sehnke et al. 2002). For example, 14-3-3 proteins regulate activities of plasma membrane H^+-ATPase (Borch et al. 2002), nitrate reductase (Huber et al. 1996), sucrose-phosphate synthase (Moorhead et al. 1999), starch synthase (Sehnke et al. 2001), chloroplast and mitochondrial ATP synthase (Bunney et al. 2001), and ion channel proteins (Booij et al. 1999, van den Wijngaard et al. 2001, de Boer 2002). Recently 14-3-3 proteins were found to interact with chaperone Hsp70 to form a guidance complex with precursors in mediating chloroplast protein import (May and Soll 2000, Sehnke et al. 2000, Jarvis and Soll 2002).

To explore the physiological functions of 14-3-3 proteins in plants, various transgenic plants with over- or under-expression of specific 14-3-3 protein genes were constructed in the last several years and novel phenotypes observed. For example, reduction of the *Arabidopsis* 14-3-3 proteins, GF14ε and GF14μ, by using antisense technology resulted in increased starch accumulation in leaves and increased growth when plants were provided with ammonium nitrate (Sehnke et al. 2001, Sehnke et al. 2002). In potato plants transformed with an overexpression or antisense construct of a 14-3-3 protein gene, the compositions of lipids, amino acids, and minerals were changed (Prescha et al. 2002, Swiedrych et al. 2002, Szopa 2002). Furthermore, overexpression of 14-3-3 proteins in potato plants leads to increase in antioxidant activity by 45% (Lukaszewicz et al. 2002) and delayed leaf senescence, whereas reduced expression of 14-3-3 protein genes (by antisense technology) in potato plants leads to early leaf senescence (Wilczynski et al. 1998).

We have studied in the function of an *Arabidopsis* 14-3-3 protein, GF14λ (Zhang et al. 1995), and found that GF14λ interacts with several proteins that include ascorbate peroxidase 3 (APX3) and ankyrin repeat-containing protein 2 (AKR2) (Zhang et al. 1997, Yan et al. 2002). Because APX3 plays an important role in protecting plants under oxidative stress and water-deficit conditions (Wang et al. 1999, Yan et al. 2003) and AKR2 is involved in both disease resistance and antioxidation metabolism (Yan et al. 2002), we thought that GF14λ might play important roles in plant antioxidation metabolism or under drought stress conditions. In an effort to further study the physiological function of *GF14λ*, we introduced *GF14λ* into cotton plants and studied whether overexpression of *GF14λ* could confer beneficial agronomic traits to this crop. Our data indi-

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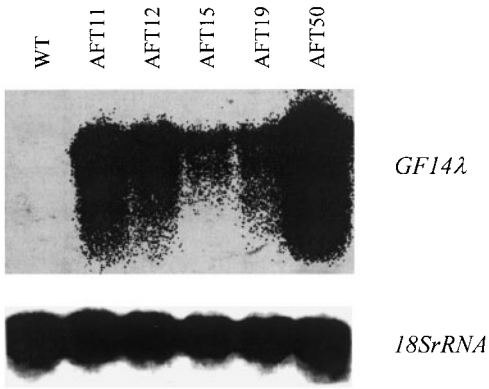

Fig. 1 RNA blot analysis of wild-type (WT) and *GF14λ*-expressing cotton plants (AFT11 to AFT50). The genes used as probes are listed on the right. A duplicate filter was used for hybridization with the *18SrRNA* gene as a loading control.

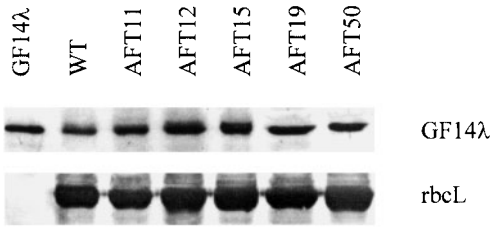

Fig. 2 Immunoblot analysis of wild-type and *GF14λ*-expressing cotton plants. Lane 1, purified GF14λ (100 ng); lane 2, wild-type cotton (WT, Coker312); lanes 3–7 (AFT11 to AFT50), five independent transgenic cotton plants. The Rubisco large subunit, rbcL, was used as the loading control.

cate that overexpression of *GF14λ* in cotton conferred a "stay-green" phenotype in transgenic cotton plants. Furthermore, these *GF14λ*-expressing cotton plants displayed increased water-stress tolerance and maintained higher photosynthetic rates under conditions of low water availability. These results indicated that it is possible to create plants with desirable agronomic traits by manipulating the expression of certain 14-3-3 protein genes.

Results

Molecular analysis of transgenic cotton plants

Twenty-six independently transformed cotton plants were obtained by *Agrobacterium*-mediated transformation (Bayley et al. 1992), and 24 of them contained the *GF14λ* transgene as demonstrated by the presence of the specific PCR products using the cauliflower mosaic virus (CaMV) 35S promoter-specific and the *GF14λ*-specific primers (data not shown). About half of those 24 lines were fertile and produced seeds. Segregation analysis of kanamycin resistance vs. sensitivity for eight independently transformed T_1 seedlings showed that five of the transgenic plants displayed a 3 to 1 ratio of resistance vs. sensitivity, two plants showed a 1 to 1 ratio, and one plant showed a 7 to 1 ratio, indicating that most of these transgenic lines possess only one T-DNA insertion. Northern blot analysis indicated that *GF14λ* transcript levels were variable in those single T-DNA insertion lines (Fig. 1), yet all these lines showed increased levels of 14-3-3 protein(s) when probed with the anti-GF14λ polyclonal antisera in the Western blot analysis (Fig. 2).

Although the GF14λ cDNA did not cross-hybridize with any cotton 14-3-3 genes, the anti-GF14λ antisera recognized the endogenous cotton 14-3-3 protein(s). This was expected because 14-3-3 proteins in eukaryotes are highly conserved (Ferl 1996). The differences in 14-3-3 protein levels between wild-type and transgenic plants shown in Fig. 2 is not likely due to the variable expression level of the endogenous cotton 14-3-3 protein(s), because every transgenic plant consistently demonstrated a higher level of 14-3-3 protein(s) than wild-type and segregated non-transgenic plants (data not shown). It appears that *GF14λ*-expressing plants have 2- to 3-fold more

Fig. 3 The "stay-green" phenotype of *GF14λ*-expressing cotton plants (#2 and #4) as compared to wild type and segregated non-transgenic plants (#1 and #3).

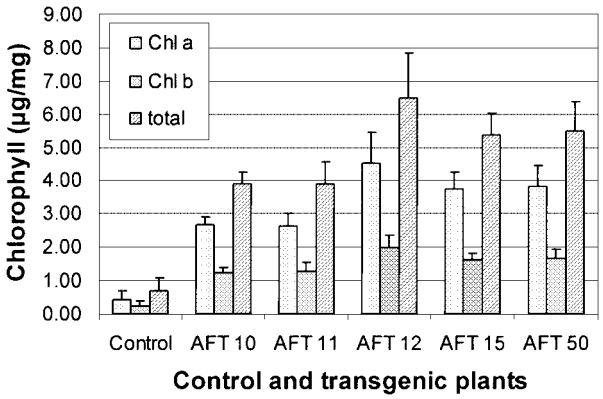

Fig. 4 Chlorophyll contents of control and transgenic plants. Values are mean ± SD (control plants, $n = 8$; transgenic plants, $n = 4$).

14-3-3 protein based on the densitometry analysis (data not shown). However, there is no correlation between the level of *GF14λ* transcript and the level of 14-3-3 protein(s) detected on Western blot. For example, lines AFT11, AFT12, and AFT50 had significantly higher *GF14λ* transcript, yet they do not seem to have much higher level of 14-3-3 protein(s) (Fig. 1, 2).

Overexpression of GF14λ leads to a "stay-green" phenotype in transgenic cotton plants

The *GF14λ*-expressing plants, their segregated non-transgenic plants, and wild-type plants were grown in a greenhouse under well-watered conditions. *GF14λ*-expressing plants displayed leaf senescence later than wild-type and segregated non-transgenic plants did (Fig. 3). The first 4–6 oldest leaves in wild-type and segregated control plants turned yellow about 2 weeks earlier than the corresponding leaves in transgenic plants did. These phenotypic differences were observed between control plants (wild-type and segregated non-transgenic plants) and every transgenic plant. We measured chlorophyll contents in the third or fourth oldest leaves from both control and *GF14λ*-expressing plants and found that indeed chlorophyll degradation occurred earlier in leaves of control plants when compared with those in transgenic plants of the same developmental stage (Fig. 4). Our data indicate that *GF14λ* expression in cotton leads to delayed leaf senescence.

GF14λ-expressing plants exhibited improved drought tolerance

In the greenhouse, we simulated a slowly developing drought as occurs in the field after a rain. During the first drought cycle, severe wilting occurred for wild type and non-expressing segregates when no transpired water was replenished (0% replenishment), while the *GF14λ*-expressing plants exhibited less wilting (Fig. 5A, C). After repeated cycles of drought and recovery, leaf damage was evident for control plants, but substantially less visible signs of injury were observed for the transgenic plants (Fig. 5B, D), indicating an improvement in drought tolerance with *GF14λ* expression.

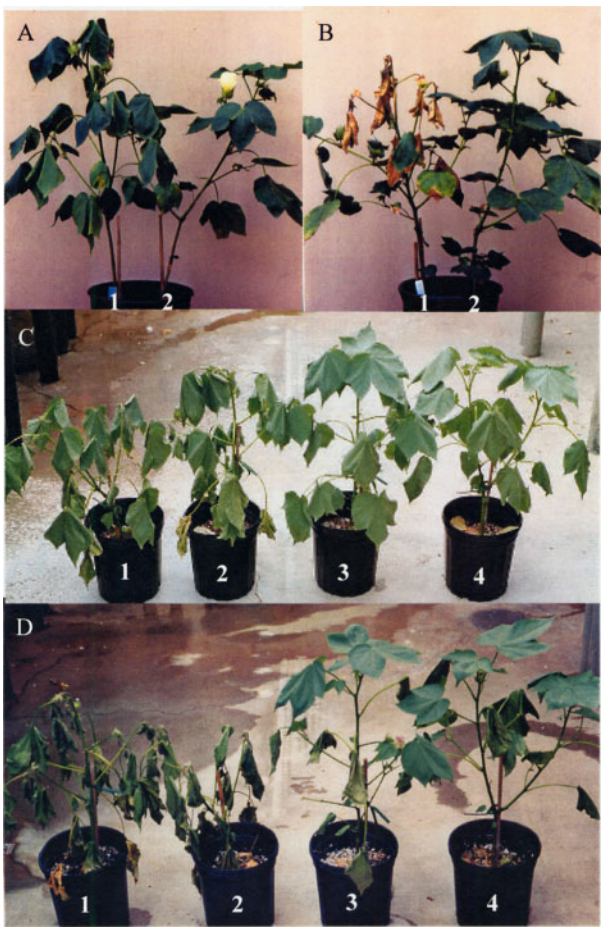

Fig. 5 *GF14λ*-expressing plants are more water-deficit tolerant. Control plants (A1, C1 and C2) wilted more than transgenic plants (A2, C3 and C4) did after one cycle of gradual water-deficit treatment. The phenotypic differences between control plants (B1, D1 and D2) and transgenic plants (B2, D3 and D4) became more evident after several cycles of water-deficit treatments.

Transgenic cotton plants maintain higher photosynthetic rates under drought conditions

Under well-watered conditions, photosynthetic rates (A), stomatal conductance (gs), and values of internal to atmospheric CO_2 concentration (Ci/Ca) were not significantly different between *GF14λ*-expressing and control plants (Fig. 6), and these values changed little during the experiment (data not shown). Although a reduction in watering to 75% of total replenishment caused a small decrease in gs, A for all plants was not affected substantially (Fig. 6A). It was not until the end of the 50% water replenishment stage that A for control plants decreased significantly ($P < 0.01$) to about 60% of that for well-watered plants in association with an 84% decline in gs (Fig. 6A, B). In contrast, the *GF14λ*-expressing plants maintained A close to the values of well-watered plants, with an average reduction of only 22% in gs. For control plants at this watering stage, the large reduction in gs resulted in a decrease in transpi-

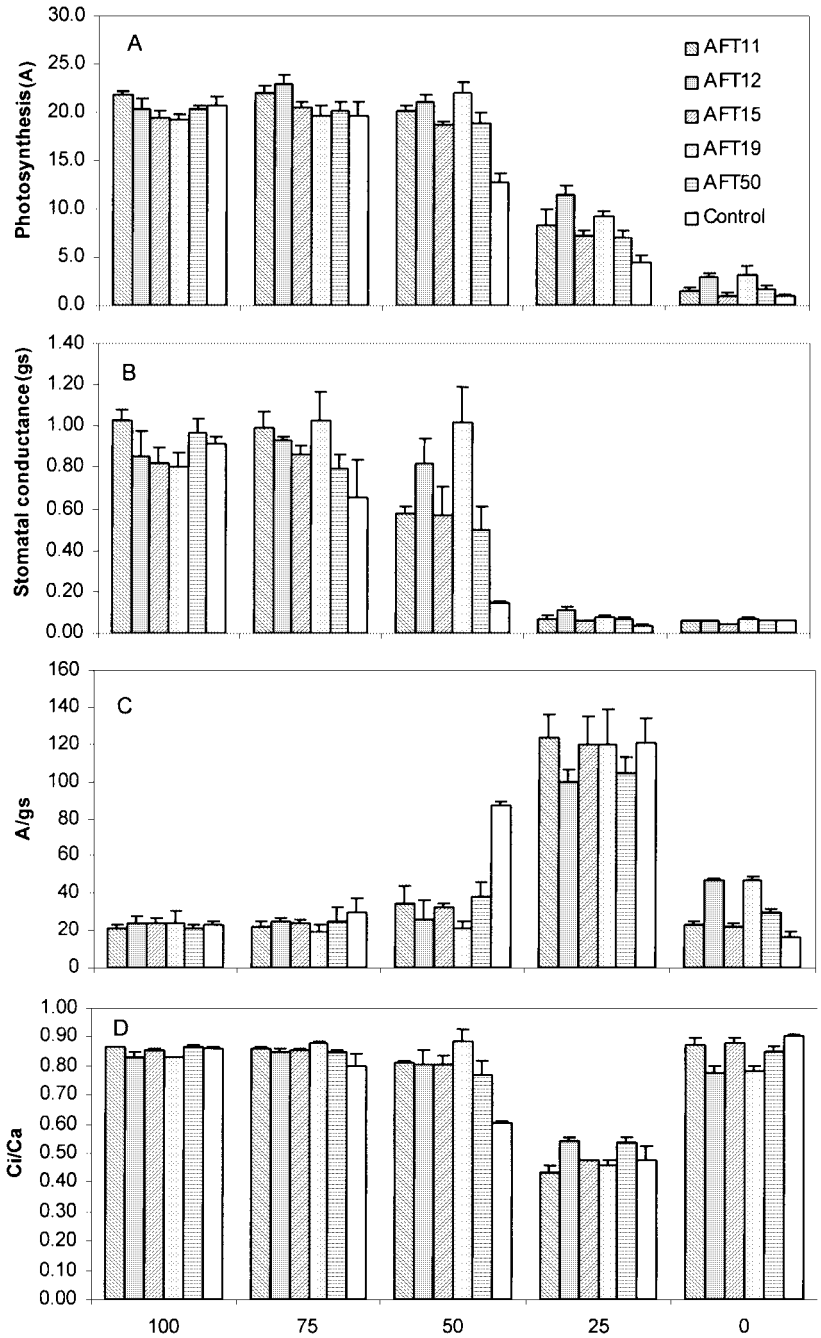

Fig. 6 Photosynthesis (A, μmol m^{-2} s^{-1}, A), stomatal conductance (gs, mmol m^{-2} s^{-1}, B), water use efficiency (A/gs, C), and intercellular to ambient CO_2 concentration ratio (Ci/Ca, D) of transgenic plants (AFT11, 12, 15, 19, 50) and non-transgenic control plants under different stages of water-deficit conditions. During the water-deficit treatments, plants were watered with 100%, 75%, 50%, 25% and 0% of the water needed to fully replenish control plants. Values are means ± SD, $n = 4$.

ration, while gs and transpiration in transgenic plants remained high (Fig. 6B, transpiration data not shown because it showed similar patterns as gs). The decreased gs in control plants led to an increase in water use efficiency (A/gs), while water use efficiency for transgenic plants remained close to that for well-watered plants (Fig. 6C). Values of Ci/Ca remained virtually unchanged at the 50% replenishment stage for transgenic plants, while they decreased for control plants (Fig. 6D).

Photosynthetic rates decreased significantly for both *GF14*λ-expressing and control plants at the 25% water replenishment stage (Fig. 6A). However, values of A for transgenic plants ranged from 30% to 60% of the values for well-watered plants. In contrast, values of A for non-transgenic control plants were only about 20% of the values for well-watered plants. Although gs was very low for all plants, A was still higher (P <0.01) for the transgenic plants than for control plants (Fig. 6A). With the exception of lines AFT12 and AFT50, A/gs was similar for transgenic and control pants (Fig. 6C). Associated with the low gs was a decline in Ci/Ca values from those values at the 50% water replenishment stage (Fig.

6D), but no significant genotypic differences were observed. When water was withheld for 3 d, A for all plants was below 20% of A for well-watered plants (Fig. 6A). Nonetheless, two transgenic lines, AFT12 and AFT19, maintained higher values of A (P <0.01) than control plants did. With no significant genotypic differences in gs, A/gs was higher for AFT12 and AFT19 than all other genotypes (Fig. 6B, C). Given the low A and gs values, it is not surprising that Ci/Ca was high at this watering stage (Fig. 6D). However, values for AFT12 and AFT19 were significantly (P <0.05) lower than those for other genotypes.

Discussion

Compared to control plants, the *GF14*λ-expressing cotton plants display a "stay-green" phenotype under well-watered conditions, which is likely due to delayed leaf senescence. This finding is similar to the observation that transgenic potato plants exhibit such a correlation between 14-3-3 protein levels and leaf senescence. Plants with reduced expression of a 14-3-3 protein gene by antisense technology exhibit early leaf senescence, whereas overexpression of a 14-3-3 protein gene leads to delayed leaf senescence (Szopa 2002, Wilczynski et al. 1998). Since 14-3-3 proteins have been shown to bind to many proteins in plant cells, it is likely that many metabolic processes have been affected in *GF14*λ-expressing cotton. For example, many enzymes involved in primary nitrogen and carbon metabolisms, such as nitrate reductase, sucrose-phosphate synthase, glutamine synthase, and glyceraldehyde-3-phosphate dehydrogenase, interact with 14-3-3 proteins (Huber et al. 1996, Moorhead et al. 1999, Cotelle et al. 2000). Binding of 14-3-3 proteins to nitrate reductase and sucrose-phosphate synthase reduces the activities of these two enzymes, which may slow down nitrogen and carbon metabolisms and elongate vegetative growth. A reduced metabolic rate is likely a major factor in delayed leaf senescence in plants (Lim et al. 2003). Another possibility is that phytohormone metabolism is altered in the *GF14*λ-expressing cotton. It is reported that 14-3-3 proteins bind to VP1 and EmBP1 proteins that mediate ABA-induced gene expression (Schultz et al. 1998). ABA plays a critical role in leaf senescence (Leung and Giraudat 1998), but it is not clear how 14-3-3 proteins might affect ABA metabolism at this time. The production of ethylene, another important phytohormone that regulates senescence in plants (Abeles et al. 1992), is 2- to 3-fold higher in transgenic potato plants with reduced 14-3-3 protein content than in control plants (Szopa 2002). Finally, it is known that 14-3-3 proteins support cell survival by antagonizing pro-apoptotic proteins, such as Bad protein, in animals (Masters et al. 2002). If GF14λ interacts and antagonizes similar pro-apoptotic proteins in plant cells, then over-expression of *GF14*λ should increase the cell lifespan and delay senescence.

Wilting during the imposition of a slowly developing drought was considerably less for *GF14*λ-expressing cotton than for control plants. This observation was interesting considering that these plants maintained a greater gs and exhibited greater rates of transpiration than control plants as drought progressed, especially at the 50% replenishment stage of watering. Thus, the transgenic plants have a greater potential for water loss than control plants during moderate drought conditions. Yet, for some reason, the transgenic plants were able to maintain greater turgor pressure and exhibited significantly less leaf damage after repeated drought and recovery cycles. Therefore, the transgenic plants were more drought tolerant than control plants. Whether *GF14*λ expression in cotton enhances osmotic adjustment, provides increased desiccation protection to leaf cells, or both, remains to be determined. It is known that plant 14-3-3 proteins regulate trehalose-6-phosphate synthase (Moorhead et al. 1999), the first enzyme in the conversion of glucose-6-phosphate to trehalose that plays a protective role during the desiccation process (de-Araujo 1996, Pilon-Smits et al. 1998).

Compared to control plants at the 50% replenishment stage, the lower A/gs and higher Ci/Ca for transgenic plants strongly suggest that their higher A was due to their ability to maintain a greater gs than control plants (Fig. 6). A major event in stomatal opening is the activation of the H^+-ATPase in the guard cell plasma membrane, which creates a driving force for passive K^+ inward permeation and opens the K^+-specific inward-rectifying channel, leading to the K^+ accumulation and water influx in the guard cell, and thereby stomatal opening (Outlaw et al. 1996, Palmgren 2001). Plant 14-3-3 proteins can activate H^+-ATPase by binding to its C-terminal regulatory domain (Jahn et al. 1997, Olsson et al. 1998, Malerba and Bianchetti 1999, Svennelid et al. 1999, Fuglsang et al. 1999), and the nine amino acid residues directly involved in binding to H^+-ATPase in plant 14-3-3 proteins (Jaspert and Oecking 2002) are completely conserved in GF14λ. Osmotic shock increases 14-3-3 proteins in the plasma membrane that form complexes with H^+-ATPase with enhanced H^+ transport in cultured tomato and sugar beet cells (Babakov et al. 2000, Kerkeb et al. 2002). This mechanism may be the explanation for the greater gs exhibited by the transgenic plants. The interaction between 14-3-3 proteins and H^+-ATPase may have also been a factor in the resistance to wilting exhibited by the transgenic plants, because turgor pressure is also regulated by the H^+-ATPase in plant cells (de Boer 2002). Since GF14λ was constitutively expressed, cells in *GF14*λ-expressing plants may have retained water better than cells in control plants as drought progressed.

At levels of watering below the 50% replenishment stage, genotypic differences in gs became negligible, yet some lines of *GF14*λ-expressing cotton plants, notably AFT12 and AFT19 at the 0% replenishment stage, continued to exhibit slightly higher values of A than did controls. In fact A/gs for these lines was also higher than that for controls. These data are suggestive of some enhancement of desiccation tolerance in those two lines. In conclusion, the "stay-green" phenotype and the increased drought tolerance with respect to leaf survival and

photosynthesis exhibited by *GF14λ*-expressing cotton plants are clearly beneficial traits for certain crops. Although an understanding of the mechanisms by which higher levels of 14-3-3 proteins affect drought tolerance is important, the results of this study indicate that the manipulation of 14-3-3 protein levels may be used to improve environmental stress tolerance in some crops.

Materials and Methods

Vector construction and cotton transformation

The full-length coding sequence of *GF14λ* (Zhang et al. 1995) was first amplified from an *Arabidopsis* cDNA library and subcloned into the dephosphorylated *Bam*HI site of the intermediate vector pRTL-2 (gift of James Carrington, Washington State University), which puts *GF14λ* under the control of the CaMV 35S promoter. Then the construct was digested with *Hin*dIII and ligated into the *Hin*dIII site of the binary vector pCGN1578 that contains the neomycin phosphotransferase gene, *NPTII*, as the selective marker (McBride and Summerfelt 1990). The overexpression construct was then introduced into the *Agrobacterium tumefaciens* strain EHA101, which was used to transform cotton according to the protocol of Bayley et al. (1992) with the following modifications. Segments of hypocotyl and cotyledon of cotton (*Gossypium hirsutum* L.) variety Coker 312 were infected for 2–3 d and then transferred to selection plates containing 50 mg liter^{-1} of kanamycin and 250 mg liter^{-1} of cefotaxime. Calluses grown in these plates were transferred to liquid medium. After suspension culture, the transformed cells were transferred to agar-solidified medium to induce somatic embryos. Once the embryos grew into seedlings, they were transferred to soil and grown in a greenhouse.

Molecular analysis of transgenic plants

Putative transgenic plants were first analyzed by using the PCR technique to detect the *GF14λ* transgene with the forward primer specific for the CaMV 35S promoter and the backward primer specific for *GF14λ*. Positive plants were then analyzed by Northern and Western blots to determine the expression level of the transgene. Cotton leaf total RNAs were isolated using the method described by Song and Allen (1997), and used in hybridization experiment following the protocol described previously (Yan et al. 2002). Cotton leaf proteins were extracted by grinding about 100 mg of mature leave tissues in a mortar in extraction buffer (50 mM $NaPO_4$ pH 7.0, 1 mM EDTA). The crude extracts were centrifuged in a microfuge at 14,000 rpm for 10 min at 4°C, and the supernatants were added to an equal volume of 2× SDS loading buffer (125 mM Tris-Cl, 2% SDS, 20% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8). Protein concentration was determined according to the Bradford method (Bradford 1976) using bovine serum albumin as standard. Proteins from *GF14λ*-expression plants and control plants were subjected to electrophoresis in a 12% SDS polyacrylamide gel. Following electrophoresis, proteins were electro-transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked by 5% non-fat milk (60 min). After washing with TTBS (20 mM Tris-Cl, pH 7.5, 140 mM NaCl, 0.05% Tween), blots were probed with polyclonal antibodies raised against *GF14λ* and the Rubisco large subunit (rbcL) for 2 h. Then blots were washed three times (10 min each) with TTBS, incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody for 30 min, and washed again as above with TTBS. Finally, the *GF14λ* and rbcL were visualized by adding substrates of alkaline phosphatase (Bio-Rad Laboratories). The sequences of primers used in PCR are as follows:

35S-1: 5′-GGGATGACGCACAATCCCACT-3′
GF14λ-1: 5′-AGAGATCCGATCACGTTTTGG-3′.

Segregation of T1 plants for kanamycin resistant and transgene

The cotyledon and first true leaf of T1 seedlings were treated with 40 mg ml^{-1} of kanamycin solution with a cotton swab. The resistant and sensitive plants were determined after one week using wild-type Coke 312 as control. PCR analysis was used to confirm the correlation between kanamycin resistance and the existence of the transgene.

Water-deficit treatment and gas-exchange analyses

About fifty seeds of the T2 generation from each of the five independently transformed cotton lines, AFT11, 12, 15, 19, and 50, were planted in a tray of soil mixture (Ball Growing on Mix, Ball Seed Co.) in the greenhouse. The temperature in the greenhouse was set at 30±2°C and natural light was used for plant growth. Fifteen to eighteen kanamycin-resistant plants and 5–6 sensitive plants per independent transgenic line were selected for PCR analysis using the CaMV 35S promoter and the *GF14λ*-specific primers to confirm the presence or absence of the transgene. These plants were used for the water-deficit experiments.

Two experimental designs for water-deficit treatment were employed. In the first design, a transgenic and a non-transgenic control plant (wild type or segregated non-transgenic line) were transplanted into each of three 11-liter pots to allow for comparisons of visible features of transgenic and control plants under the same soil water status. In the second design, transgenic and control plants were planted in separate pots for gas-exchange analyses. Ten to twelve transgenic plants of each line were transferred to 4-liter pots with one plant per pot. Fifteen segregated, non-transgenic plants randomly selected from different transgenic lines were also transferred to pots as controls. These plants were watered three times a day and fertilized once a week with Hoagland's solution until plants reached about 40 cm in height. Eight plants with similar height were chosen from each transgenic line and controls for the experiment. The eight plants were divided into two sets. One set of four plants was used for the water-deficit treatments, and the other set was used for well-watered controls. The first fully developed leaf of each plant was used for gas-exchange measurements. Growth conditions were the same for both sets of plants, except the watering treatments.

The gradual development of water deficit was accomplished as described previously (Yan et al. 2003). In brief, water deficit was developed in the greenhouse during the summer by progressively reducing the amount of water added on a daily basis. The treatment schedule consisted of 3 d at 100% replenishment of water lost, followed by 3 d at each reduced watering regime (75%, 50%, 25%, 0% replenishment of the water lost by well-watered plants). In the first experimental design, plants were fully re-watered in the morning after 3 d at 0% replenishment, and then the imposition of a gradually developing water-deficit was repeated. When the two plants in the same pot showed discernible phenotypic differences after several cycles of water-deficit treatment, they were photographed. For the second experimental design, in the first cycle of water-deficit treatment, gas-exchange measurements to determine rates of net photosynthesis, stomatal conductance, transpiration and the ratio of the intercellular to ambient CO_2 concentration (Ci/Ca) were performed on the third day of each watering stage for both water-deficit and well-watered plants using a portable photosynthesis system (Model LI6400, Li-Cor, Inc.). The experiment was repeated twice with similar results. The data from the second replication are presented.

Chlorophyll determination

Extracts of the third or fourth true leaves of control and transgenic plants on day 132 after germination were analyzed for chlorophyll contents using the method of Lichtenthaler and Wellburn (1955).

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