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Regulated Expression of an Isopentenyltransferase Gene (*IPT*) in Peanut Significantly Improves Drought Tolerance and Increases Yield Under Field Conditions

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Isopentenyltransferase (IPT) is a critical enzyme in the cytokinin biosynthetic pathway. The expression of *IPT* under the control of a maturation- and stress-induced promoter was shown to delay stress-induced plant senescence that resulted in an enhanced drought tolerance in both monocot and dicot plants. This report extends the earlier findings in tobacco and rice to peanut (*Arachis hypogaea* L.), an important oil crop and protein source. Regulated expression of *IPT* in peanut significantly improved drought tolerance in both laboratory and field conditions. Transgenic peanut plants maintained higher photosynthetic rates, higher stomatal conductance and higher transpiration than wild-type control plants under reduced irrigation conditions. More importantly, transgenic peanut plants produced significantly higher yields than wild-type control plants in the field, indicating a great potential for the development of crops with improved performance and yield in water-limited areas of the world.

Keywords: Crop improvement • Drought resistance • Isopentenyltransferase • P_{SARK} • Peanut • Transgenic plants.

Abbreviations: BA, N^6-benzyladenine; IPT, isopentenyltransferase; MS, Murashige and Skoog; SIM, shoot induction medium; TD-NMR, time domain nuclear magnetic resonance.

Introduction

Environmental stresses such as drought, salinity and extreme temperatures are major factors that cause significant losses in agricultural productivity worldwide (Boyer 1982, Boyer and Westgate 2004, Barnabas et al. 2008). Water availability is the major limiting factor for food production in many countries, with agriculture consuming about 75% of water in developed countries and close to 90% of water in many developing countries (Boyer 1982, Pennisi 2008). The expected increase in human population with the concomitant demands in food and urban water use and the increased weather variability due to global climate change will require a more efficient production of food crops under limited input farming systems (Pennisi 2008, Battisti and Naylor 2009, FAOUN 2010, Long and Ort 2010). The primary challenge in agricultural sciences today is to develop technologies that will increase food production and the sustainability of agriculture under environmentally limiting conditions.

Over the last 10 years, many genes that display altered expression patterns in response to environmental stresses have been identified (Bray 2004, Shinozaki and Yamaguchi-Shinozaki 2007) and the functions of some of these genes have been studied in detail (Vinocur and Altman 2005, Lemaux 2008, Lemaux 2009, Mittler and Blumwald 2010). Several genes that confer drought tolerance have been tested in the field for many years and a few are waiting for the approval of commercial release at US federal regulatory agencies (Castiglion et al. 2008, Yang et al. 2010).

The first group of genes selected for biotechnological application include genes encoding transcription factors that control gene expression under stress conditions (Century et al. 2008). For example, overexpression of a maize gene, i.e. *ZmDREB2A*, in Arabidopsis led to increased drought and heat tolerance in transgenic plants (Qin et al. 2007), because DREB2A regulates expression of both dehydration-inducible genes and heat shock-related genes (Sakuma et al. 2006). This gene appears to have great value in developing heat- and drought-tolerant crops in the future. In addition to the DREB/CBF class of transcription factors, of which ZmDREB2A is a member (Liu et al. 1998), other classes of transcription

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factors were also found to be effective in conferring drought tolerance (Umezawa et al. 2006, Shinozaki and Yamaguchi-Shinozaki 2007, Yang et al. 2010). For example, overexpression of a stress-inducible gene, *SNAC1*, significantly improved drought tolerance in transgenic rice and increased seed yields by 23–34% under severe drought conditions in the field (Hu et al. 2006). Overexpression of a maize transcription factor gene, *ZmNF-YB2*, conferred increased drought tolerance in transgenic corn plants in the field as well (Nelson et al. 2007). The *ZmNF-YB2*-overexpressing plants demonstrated improved performance in photosynthesis and stomatal conductance, and maintained higher chlorophyll content and lower leaf temperatures under water deficit conditions (Nelson et al. 2007).

Besides progress made in expressing stress-related transcription factor genes in transgenic plants, overexpressing genes that are involved in stress signal transduction pathways or overexpressing functional protein genes also resulted in increased drought tolerance in transgenic plants (Lemaux 2008, Lemaux 2009). For example, overexpression of a tobacco protein kinase gene *NPK1* led to significantly increased tolerance against freezing, heat and salt in transgenic plants (Kovtum et al. 2000). The constitutive expression of the kinase domain of NPK1 could improve drought tolerance in transgenic maize (Shou et al. 2004). Furthermore, when a rice ortholog of *NPK1* was expressed under the control of a water deficit-inducible promoter, it improved yield production by 23% under rain-free field conditions (Xiao et al. 2009). Another example of manipulating stress signaling pathways to increase drought tolerance in plants is to increase the sensitivity of plants to the plant stress hormone ABA. Wang et al. (2005, 2009) demonstrated that down-regulation of farnesyltransferase activity by antisense or RNAi (RNA interference) silencing led to increased sensitivity to ABA, inducing smaller stomatal apertures under drought conditions, which led to reduced water loss and increased drought tolerance (Wang et al. 2005, Wang et al. 2009).

Several reports have indicated that changes in hormone homeostasis, brought about by the expression of *IPT* (isopentenyltransferase, a key enzyme in the biosynthesis of cytokinin), under the control of P_{SARK}, a maturation- and stress-induced promoter, resulted in enhanced drought tolerance (Rivero et al. 2007, Rivero et al. 2009, Rivero et al. 2010). Increased cytokinin levels led to increased drought tolerance in tobacco plants (Rivero et al. 2007), enhanced photorespiration (Rivero et al. 2009) and the protection of the photosynthetic apparatus under stress (Rivero et al. 2010). The notion of a cytokinin-induced change in hormone homeostasis during stress with a concomitant enhanced drought tolerance was supported recently in transgenic rice plants expressing P_{SARK}::*IPT* (Peleg et al. 2011). The transgenic rice plants displayed relatively low levels of yield penalty after the plants were exposed to a severe drought episode during flower development, the most stress-sensitive developmental stage.

Although the beneficial role of cytokinin production during stress has been demonstrated both in dicot (Rivero et al. 2007) and monocot plants (Peleg et al. 2011), these studies were conducted in greenhouse conditions. Here we present evidence of the role of enhanced cytokinin in protecting plants against the deleterious effects of water deficit under field conditions. We show that transgenic peanut plants expressing P_{SARK}::*IPT* are significantly more drought tolerant than wild-type control plants growing under restricted water conditions in the field. The transgenic peanut plants produced between 51 and 65% more seeds than wild-type controls when tested in two consecutive years under field conditions. Our data support the notion that the production of cytokinins offers an effective strategy for the production of drought-resistant plants with improved yield under stress.

Results

Introduction of *IPT* into peanut

The P_{SARK}::*IPT* construct (Rivero et al. 2007) was introduced into the peanut genome (New Mexico Valencia A variety) by using the *Agrobacterium*-mediated transformation method described by Sharma and Anjaiah (2000). Transgenic peanut plants were identified by PCR amplification of the *IPT* transgene using genomic DNA isolated from leaves of putative transgenic plants (T_1 generation). Approximately 50% of the >60 independent lines tested contained the *IPT* transgene (**Fig. 1A**). Plants that were positive in the PCR analysis were subjected to a mild drought stress for 5 d to induce expression of the *IPT* transgene through the action of the drought-inducible *SARK* promoter (Peleg et al. 2011). The presence of the *IPT* transcript was detected by RNA blot analysis (**Fig. 1B**). Genomic DNAs from four transgenic lines were isolated and subjected to DNA blot analysis. Because the restriction enzyme *Hin*dIII does not cut within the *IPT* transgene and only one band was found in

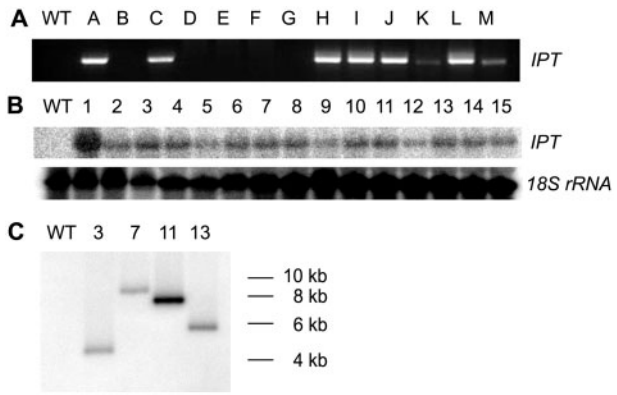

Fig. 1 Molecular analysis of transgenic peanut plants. (A) PCR analysis of transgenic plants. WT, wild-type; lanes A–M, 13 independent putative transgenic peanut plants. (B) RNA blot analysis of transgenic plants. Lanes 1–15, transgenic peanut plants that tested positive in the PCR experiment. 18S rRNA was used as the RNA loading control. (C) DNA blot analysis of four transgenic peanut plants. Lanes WT, 3, 7, 11 and 13, wild-type and four independent transgenic lines.

these four lines (Fig. 1C), we concluded that these four lines were all single insertion transgenic lines. These single insertion lines were used for comparative study in the laboratory and in the field.

Expression of *IPT* in peanuts enhances drought tolerance

Under full irrigation conditions, wild-type controls and three independent transgenic lines (3, 7 and 11) displayed no significant differences in biomass (Fig. 2) or in photosynthetic performance (Fig. 3) after these plants were grown in a growth chamber for 2 months. To test if P_{SARK}::*IPT* transgenic peanut plants are more drought tolerant, we conducted reduced irrigation experiments in growth chambers. Wild-type controls and the three independent transgenic lines were first grown under normal growth conditions for 15 d and, during this period, all plants were roughly the same size and did not display phenotypic differences (Fig. 4A). Watering was then stopped for 15 d. On day 31, plants were watered every 6 d with only a quarter of the water amounts used normally (a previous experiment determined that 600 ml of water would completely soak the 11 liter pot without water leaking out of the pot). Following a further 45 d of growth under the reduced irrigation condition, P_{SARK}::*IPT*-peanut plants were bigger and bushier than the wild-type plants (Fig. 4B). The root systems of the P_{SARK}::*IPT*-peanut plants were larger than those of wild-type peanut plants (Fig. 4C). The fresh shoot weight and the fresh root weight of the P_{SARK}::*IPT*-plants were significantly higher than those of control plants (84 and 188%, respectively, Fig. 5A, B). Similar results were obtained for dry shoot weight and dry root weight (61 and 124% increase, respectively, Fig. 5C, D).

The phenotypic differences displayed by wild-type and P_{SARK}::*IPT*-peanut plants grown under water-limiting conditions suggested dramatic differences in plant growth and development between the two genotypes under drought. The photosynthetic performance of wild-type and P_{SARK}::*IPT*-plants under reduced irrigation conditions was measured. The photosynthetic data were collected after the plants were grown under reduced irrigation condition for 45 d. P_{SARK}::*IPT*-plants displayed photosynthetic rates that were at least 2-fold higher than those of the wild-type plants (Fig. 6A). The transpiration rates of P_{SARK}::*IPT*-plants were also significantly higher than that of control plants (about twice as high, Fig. 6B), probably due to the higher stomatal conductance of the transgenic plants (60% higher, Fig. 6C).

The water deficit treatment caused considerable biomass reductions among all plants, but the penalty was significantly more severe in wild-type plants than that in P_{SARK}::*IPT*-peanut plants (81% reduction in wild-type vs. 65% reduction in P_{SARK}::*IPT*-peanut plants). In comparing the photosynthetic performance of these two genotypes, the water deficit treatment also caused a larger penalty in wild-type plants than in P_{SARK}::*IPT*-peanut plants (77.5% reduction vs. 36.4% reduction, respectively). It is clear that the higher photosynthetic rates observed in P_{SARK}::*IPT*-peanut plants are responsible for a larger biomass in all P_{SARK}::*IPT*-peanut plants.

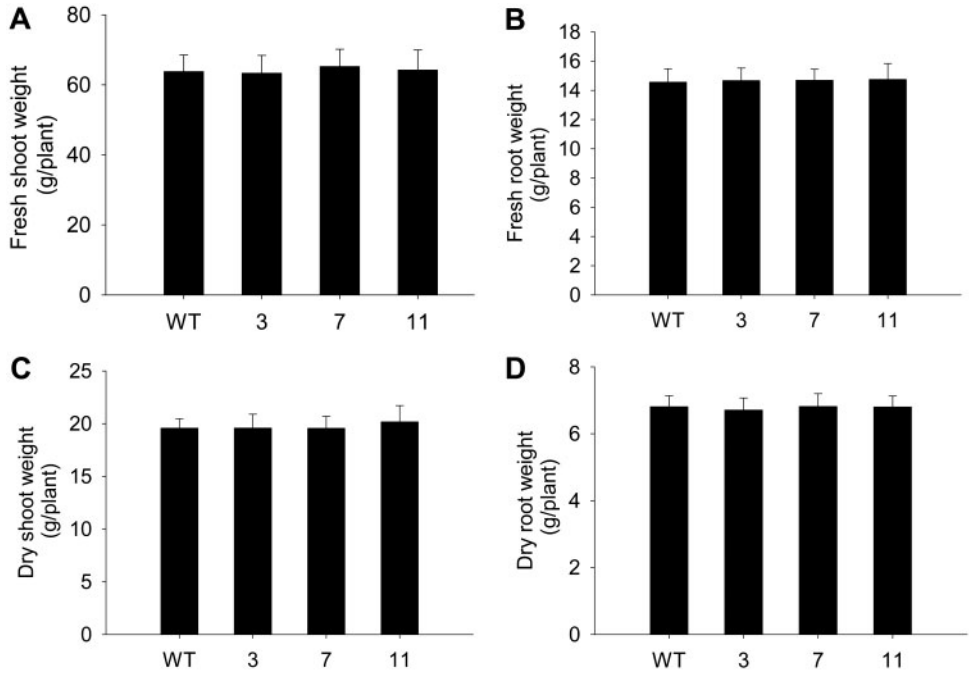

Fig. 2 Biomass analysis of peanut plants grown in a growth chamber under normal irrigation conditions for 60 d. (A) Fresh shoot weight of wild-type and transgenic peanut plants. (B) Fresh root weight of peanut plants. (C) Dry shoot weight of peanut plants. (D) Dry root weight of peanut plants. WT, wild-type; 3, 7 and 11, three independent transgenic peanut plants. Bar, standard error; $n = 6$ for each line.

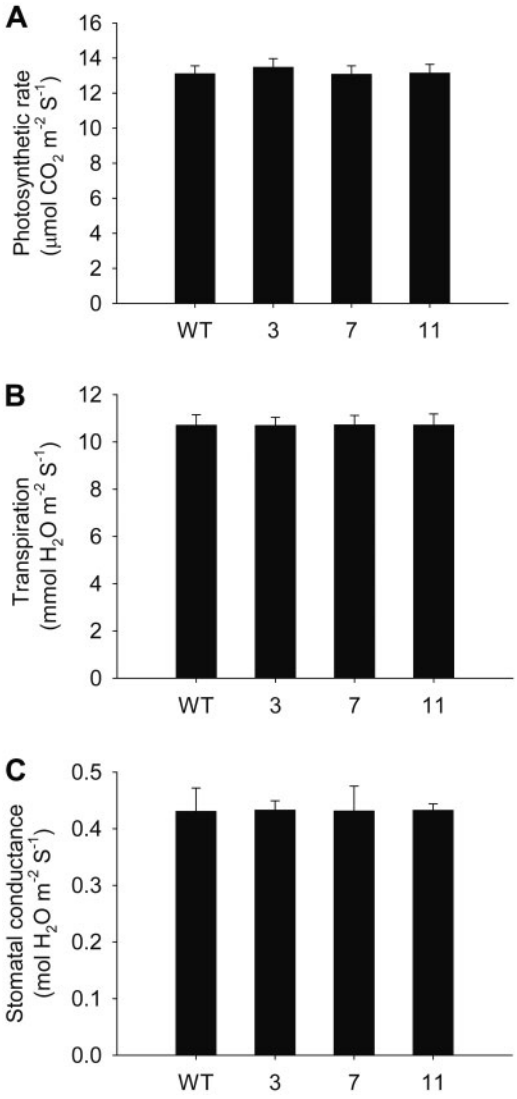

Fig. 3 Photosynthetic performance of peanut plants grown in a growth chamber under normal irrigation conditions for 60 d. (A) Photosynthetic rates of wild-type and transgenic peanut plants. (B) Transpiration rates of wild-type and transgenic peanut plants. (C) Stomatal conductance of wild-type and transgenic peanut plants. WT, wild-type; 3, 7 and 11, three independent transgenic peanut plants. Bar, standard error; $n = 6$ for each line.

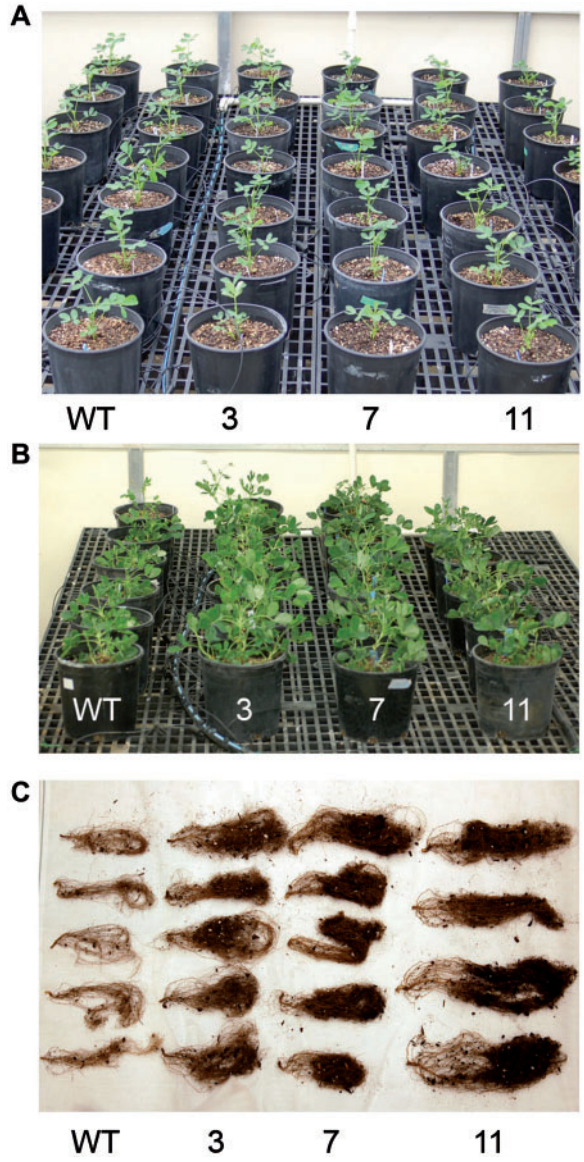

Fig. 4 Phenotypes of transgenic plants before and after drought treatment in a growth chamber. (A) Fifteen-day-old peanut plants under normal growth conditions. (B) Peanut plants under reduced irrigation conditions for 60 d. (C) Root phenotype of peanut plants under reduced irrigation condition for 60 d. WT, wild-type; 3, 7 and 11, three independent transgenic plants.

Growth of P_{SARK}::*IPT*-peanut plants under water-limiting conditions in the field

To analyze the performance of the P_{SARK}::*IPT*-peanut plants in the field under water-limiting conditions, wild-type and three independent P_{SARK}::*IPT*-peanut lines 3, 7 and 11 were grown at the USDA-ARS Experimental Farm in Lubbock, Texas in the summer of 2009. These plants were treated as grown under dryland conditions, and were flood-irrigated when a long period of no rain conditions occurred. No effort was made to measure gas exchange parameters with these plants, but genomic DNAs were prepared from each plant for PCR analysis and the final yield data were collected at the end of the growth season. In 2010, two more lines were tested in addition to the four lines tested 1 year earlier: a non-transgenic line (regenerated from tissue culture) and another independent transgenic plant line (i.e. 13) at the USDA-ARS Experimental Farm in Lubbock, Texas. These plants were treated differently: reduced irrigation (19 mm per week, low irrigation group) and regular irrigation (38 mm per week, high irrigation group). Photosynthetic performance of the plants in the low irrigation group was analyzed in the middle of August. Our data indicated that the photosynthetic rates of the transgenic plants were

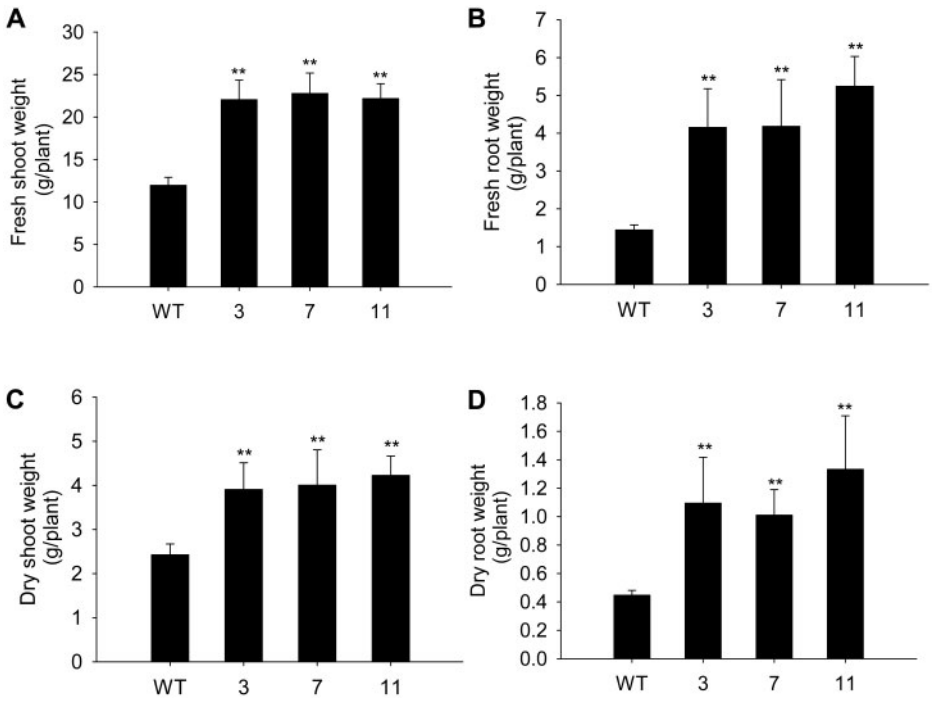

Fig. 5 Biomass analysis of peanut plants grown in a growth chamber under reduced irrigation conditions for 60 d. (A) Fresh shoot weight of wild-type and transgenic peanut plants. (B) Fresh root weight of peanut plants. (C) Dry shoot weight of peanut plants. (D) Dry root weight of peanut plants. WT, wild-type; 3, 7 and 11, three independent transgenic peanut plants. Bar, standard error; **, significant at 1%; $n = 6$ for each line.

generally higher than those of control plants (**Fig. 7A**), but the differences, although significant, were smaller than what was observed for these plants under reduced irrigation conditions in the growth chamber. Similar data were obtained for transpiration rates and stomatal conductance (**Fig. 7B, C**).

The transgenic peanut plants in the low irrigation group grew faster after germination and quickly established a larger area within 2 months after planting in the field (**Fig. 8A**). This difference remained until the end of the growth season because the above-ground dry biomass from transgenic plants was significantly higher than that of wild-type controls (**Fig. 8B**). In 2009, we observed yield increases between 35 and 80% from the three independent P_{SARK}::*IPT*-peanut lines, with an average increase of 65% (**Fig. 9A**); whereas in 2010, we found that the transgenic plants in the low irrigation group produced 35–79% more peanuts than wild-type plants, with an average increase of 51% (**Fig. 9B**). No statistical differences in yield between the wild-type and transgenic lines were observed when the plants were grown under high irrigation conditions (**Fig. 9C**). The low irrigation-induced yield penalties were 62% in the wild-type plants and 45, 30, 52 and 46% in transgenic lines 3, 7, 11 and 13, respectively. Although field conditions differed considerably between 2009 and 2010 (**Table 1**), our results showed that the transgenic plants grown under low irrigation conditions outperformed the wild-type plants in both years.

There were no differences in the oil content between wild-type control and transgenic peanut plants (**Table 2**). The contents of major fatty acids in peanut, palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) were also similar between wild-type control and transgenic peanut plants (**Table 2**). The minor fatty acids such as stearic acid, gadoleic acid and behenic acid varied a little between wild-type control and transgenic peanut plants; however, this difference in minor amino acid composition was also found between the wild-type and a tissue culture regenerated line (**Table 2**). It is clear that *IPT* expression in peanut does not affect oil content and major fatty acid composition.

Discussion

We have shown that expression of P_{SARK}::*IPT* under reduced irrigation conditions improves drought tolerance in transgenic peanut plants, which is consistent with what Rivero et al. (2007) and Peleg et al. (2011) had demonstrated in tobacco and rice plants, respectively. The *IPT*-expressing peanut plants are visibly much larger in size than wild-type plants under reduced irrigation in a growth chamber (**Fig. 4B**) and in dryland conditions in the field (**Fig. 8A**). Their biomass is also significantly higher than that of wild-type plants in both growth chamber and field conditions (**Figs. 5**, **8B**). These differences are probably due to higher photosynthetic rates observed with the *IPT*-expressing peanut plants in these conditions (**Figs. 6**, **7**). The higher yields in the transgenic peanut plants correlate well with the increases in photosynthesis, stomatal conductance and transpiration, supporting the notion of a

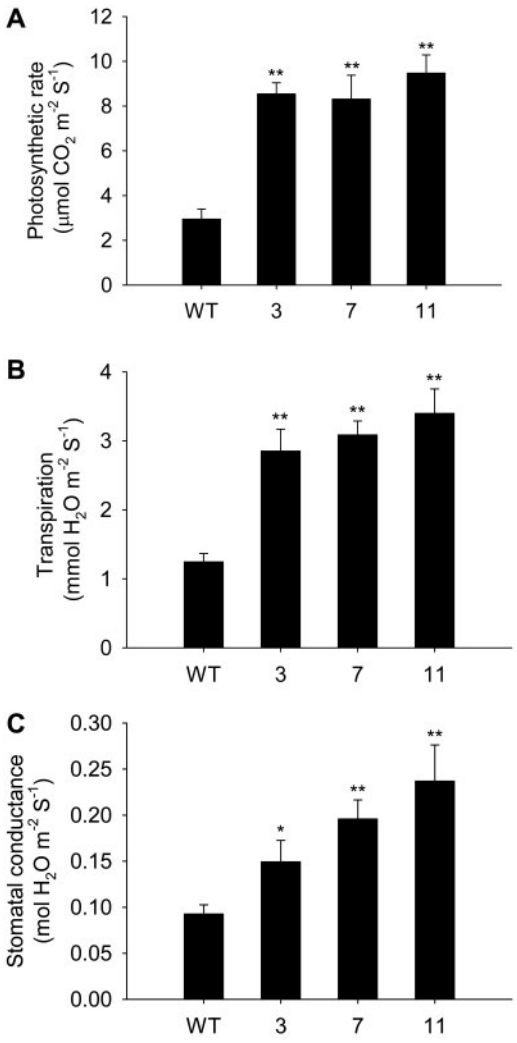

Fig. 6 Photosynthetic performance of peanut plants grown in a growth chamber under reduced irrigation conditions for 60 d. (A) Photosynthetic rates of wild-type and transgenic peanut plants. (B) Transpiration rates of wild-type and transgenic peanut plants. (C) Stomatal conductance of wild-type and transgenic peanut plants. WT, wild-type; 3, 7 and 11, three independent transgenic peanut plants. Bar, standard error; *, significant at 5%; **, significant at 1%; $n = 6$ for each line.

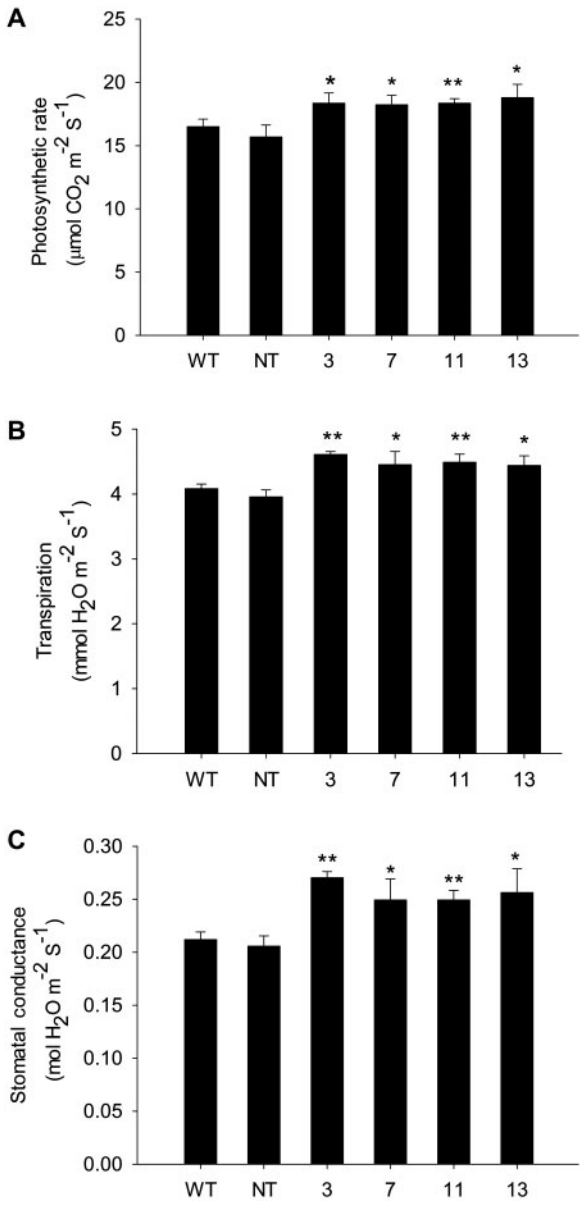

Fig. 7 Photosynthetic performance of field-grown peanut plants under field conditions. (A) Photosynthetic rates of controls and transgenic peanut plants. (B) Transpiration rates of controls and transgenic peanut plants. (C) Stomatal conductance of controls and transgenic peanut plants. WT, wild-type; NT, non-transgenic line originating from tissue culture; 3, 7, 11 and 13, four independent transgenic peanut plants. Bar, standard error; *, significant at 5%; **, significant at 1%; $n = 12$ for each line.

cytokinin-mediated protection of photosynthesis in the transgenic plants (Rivero et al. 2009, Rivero et al. 2010). This report is the first example showing that *IPT*-expressing transgenic plants could produce significantly higher yields (58% more seeds on average based on the data of 2 years) in dryland conditions in the field (**Fig. 9**), indicating the potential for this approach to be be used to improve crop production in water-limited areas of the world.

During the peanut transformation, we observed a higher rate of abnormal young seedlings in tissue culture with the P_{SARK}::*IPT* construct, an indication that the *IPT* gene was probably expressed during the regeneration process, which affected the auxin/cytokinin ratio and led to abnormal seedlings that could not grow into fertile plants. However, when transgenic plants were obtained from tissue culture, most of them looked normal and behaved like wild-type plants under normal growth conditions, suggesting that the P_{SARK}::*IPT* construct does not affect transgenic peanut plants in negative ways. The *IPT* gene under the control of a senescence-inducible promoter from a gene called *SAG12* (Gan and Amasino 1995) was introduced into wheat, but no grain yield increase was observed

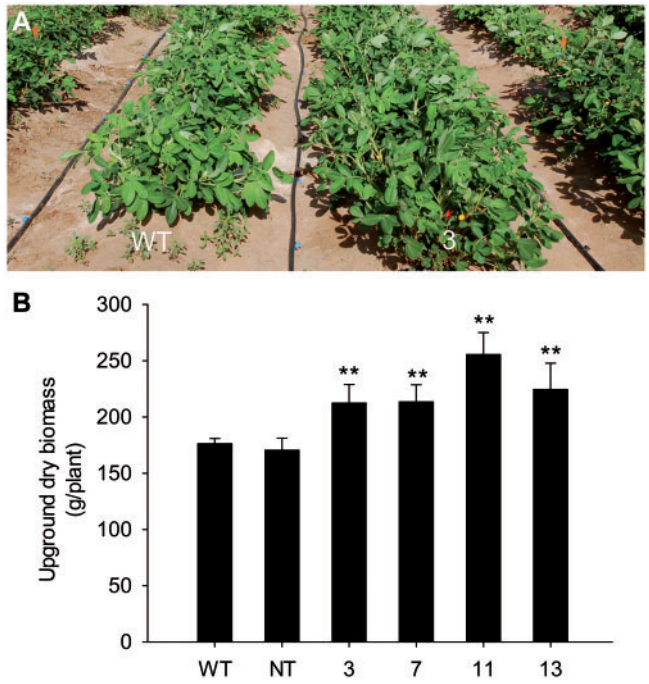

Fig. 8 Phenotype and biomass of wild-type and transgenic peanut plants under field conditions. (A) Phenotype of wild-type and transgenic peanut plants in the field. (B) Biomass of wild-type and transgenic peanut plants in the field at the end of the growth season. WT, wild-type; NT, non-transgenic line originating from tissue culture; 3, 7, 11 and 13, four independent transgenic peanut plants. Bar, standard error; **, significant at 1%; $n = 40$ for each line.

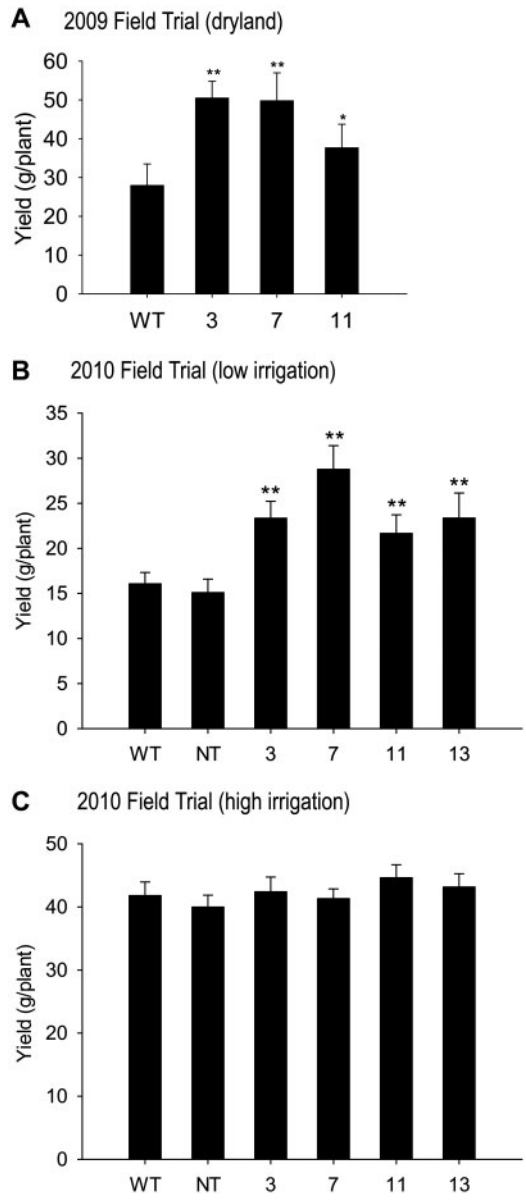

Fig. 9 Peanut yields from wild-type, non-transgenic and transgenic peanut plants under field conditions. (A) Yields from the 2009 field trial. (B) Yields from the low irrigation group in the 2010 field trial. (C) Yields from the high irrigation group in the 2010 field trial. WT, wild-type; NT, non-transgenic line originating from tissue culture; 3, 7, 11 and 13, four independent transgenic peanut plants. Bar, standard error; *, significant at 5%; **, significant at 1%; $n = 40$ for each line.

(Sýkorová et al. 2008). The *IPT*-expressing wheat plants did display delayed leaf senescence, increased cytokinin content, nitrate influx and nitrate reductase. This is interpreted to mean that the delayed senescence by the *SAG12::IPT* construct might also delay metabolite translocation from leaves to developing grains, which in turn affects grain yield (Sýkorová et al. 2008). Zhang et al. (2010) recently reported that the *SAG12::IPT* construct was introduced into cassava, and they observed increased drought tolerance and delayed senescence in the transgenic cassava plants. Again, no increased yield was observed, perhaps due to a similar reason to that in the case in wheat. Other studies with *SAG12::IPT* transgenic plants also did not report significant improvement in yields (Ma 2008). It is clear that the promoters used in these experiments play a critical role in the impact of overexpression of *IPT* on the yield of transgenic plants.

Bhatnagar-Mathur et al. (2007) introduced the Arabidopsis drought-inducible transcription factor gene *AtDREB1A* into peanut and observed increased transpiration efficiency under drought conditions. However, the report did not show whether the drought-inducible *AtDREB1A* expression in peanut plants could improve peanut yield under water deficit or reduced irrigation conditions in the field or in laboratory conditions. The *AtDREB1A*-expressing peanut plants displayed reduced stomatal conductance under drought conditions, which is in contrast to our data showing that all *IPT*-expressing peanut plants have higher stomatal conductance (**Figs. 6**, **7**). Perhaps this is due to different mechanisms that impact drought tolerance in transgenic plants. The DREB1A transcription factor functions in ABA-independent signaling pathways, which regulates expression of genes that are involved in drought, salt and cold stresses (Shinozaki and Yamaguchi-Shinozaki 2007), whereas the expression of *IPT* driven by the *SARK* promoter leads to altered hormone metabolism, especially increased expression of brassinosteroid-related genes and repression of

jasmonate-related genes (Rivero et al. 2010, Peleg et al. 2011). This differential expression of genes resulted in hormonal cross-talk and robust root development [i.e. 2–3 times more root biomass in transgenic peanut plants (**Fig. 5**)], which could increase the water-absorbing capacity of transgenic plants, allowing higher stomatal conductance even under drought conditions that ensures higher CO_2 supply to leaf tissues and therefore a higher photosynthetic rate. Consequently, P_{SARK}::*IPT*-peanut plants outperform wild-type plants under reduced irrigation conditions and in field conditions. The seeds from P_{SARK}::*IPT*-peanut plants are normal in terms of oil content and composition (**Table 2**), indicating that no negative changes have been introduced into transgenic peanut and the nutritional value of transgenic peanut seeds is still the same. In summary, we have shown that regulated expression of *IPT* can indeed significantly improve drought tolerance in transgenic peanut plants and *IPT* can be an excellent gene for engineering other crops for increased drought tolerance.

Materials and Methods

Peanut transformation

The *Agrobacterium* strain EHA104 harboring the binary vector P_{SARK}::*IPT* (Rivero et al. 2007) was used for peanut transformation. A single colony of the *Agrobacterium* strain was grown overnight at 28°C in LB medium (pH 7.2) containing 100 mg l^{-1} rifampicin, 50 mg l^{-1} streptomycin and 50 mg l^{-1} kanamycin, to late log phase (OD_{600} = 1.0–1.5). Bacterial cells were collected by centrifugation, resuspended in fresh MS medium (Murashige and Skoog 1962) and left on the shaker for at least 1 h. Mature seeds of peanut (*Arachis hypogaea* L., New Mexico Valencia A) were surface-sterilized by rinsing with 70% (v/v) ethanol for 1 and 10 min with 0.1% (w/v) aqueous mercuric chloride followed by several washes in sterile water. Seeds were left in sterile water for 2 h before use. The seed coat and embryo axis were removed and each cotyledon was cut in half vertically to obtain the cotyledon explants. Freshly prepared cotyledon explants were placed in the *Agrobacterium* cell suspension for 10 min, and then air dried briefly. *Agrobacterium*-inoculated explants were blotted dry, and incubated on shoot induction medium [SIM, i.e. MS medium supplemented with 10 mg l^{-1} N^6-benzyladenine (BA), 1 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 2 g l^{-1} phytagel, pH 5.8] for 3 d at 28°C in darkness.

Inoculated explants were transferred to fresh SIM supplemented with 300 mg l^{-1} cefotaxime, and cultured with a 16 h photoperiod at a light intensity of 200 µmol photons m^{-2} s^{-1} for an additional 2 weeks. Explants displaying multiple shoot buds were subcultured onto SIM with 250 mg l^{-1} cefotaxime and 125 mg l^{-1} kanamycin for 2 weeks to initiate selection and enrichment of transformed cells. Subsequently, proximal parts of the explants containing multiple adventitious shoot buds were excised and transferred to shoot elongation medium (MS medium supplemented with 0.5 mg l^{-1} BA, 30 g l^{-1} sucrose, 2 g l^{-1} phytagel, pH 5.8) with 250 mg l^{-1} cefotaxime and 125 mg l^{-1} kanamycin for 2–3 subcultures of 3 weeks duration each for further shoot development and proliferation. Elongated shoots were cut and rooted on root induction medium (MS medium supplemented with 1 mg l^{-1} 1-naphthaleneacetic acid, 30 g l^{-1} sucrose, 2 g l^{-1} phytagel, pH 5.8).

Table 1 Weather information for the Lubbock area in 2009 and 2010: average rainfall and temperature change from May to November

Time	2009		2010	
	Rainfall (in)	Temperature (°F) (high/low)	Rainfall (in)	Temperature (°F) (high/low)
May	0.68	82.7/55.7	1.14	82.5/56.5
June	2.44	92.9/66.2	2.55	93.9/68.0
July	1.69	93.7/68.5	7.14	86.5/68.2
August	0.47	94.6/67.2	1.33	93.2/67.3
September	2.46	83.3/57.5	0.93	87.9/61.8
October	0.78	72.0/44.3	2.61	79.4/47.9
November	0.13	69.2/36.2	0.07	66.9/34.7

Plant growth conditions in growth chamber

Seeds of wild-type and P_{SARK}:: *IPT*- plants were sown directly in 11 liter pots filled with pro-mix BX peat moss, perlite and

Table 2 The oil content and fatty acid composition of wild-type and P_{SARK}::*IPT*-transgenic peanut plants grown under irrigated conditions in 2010

Sample	Oil content	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:0}$	$C_{20:1}$	$C_{22:0}$	$C_{24:0}$
WT	47.77 ± 0.90	10.70 ± 0.26	2.82 ± 0.35	38.90 ± 1.46	39.46 ± 1.60	1.51 ± 0.15	1.29 ± 0.07	3.51 ± 0.36	1.52 ± 0.18
NT	47.59 ± 1.14	10.39 ± 0.36	2.19 ± 0.18*	39.29 ± 1.53	39.86 ± 1.74	1.41 ± 0.05	1.56 ± 0.15**	3.79 ± 0.48	1.98 ± 0.40*
3	46.76 ± 0.51	11.17 ± 018*	2.78 ± 0.16	39.04 ± 0.87	39.81 ± 0.75	1.41 ± 0.08	1.27 ± 0.12	2.49 ± 0.29**	1.71 ± 0.25
7	47.98 ± 0.44	10.81 ± 0.58	2.31 ± 0.29*	40.07 ± 1.23	38.93 ± 1.06	1.31 ± 0.12*	1.34 ± 0.03	3.08 ± 0.33	1.89 ± 0.21*
11	47.26 ± 0.54	10.85 ± 0.39	3.29 ± 0.47	40.25 ± 0.80	37.92 ± 1.59	1.58 ± 0.20	1.17 ± 0.03*	2.96 ± 0.34*	1.75 ± 0.12*
13	47.74 ± 0.55	10.52 ± 0.52	2.55 ± 0.26	41.40 ± 2.11	39.93 ± 2.63	1.40 ± 0.17	1.39 ± 0.10	3.30 ± 0.48	1.46 ± 0.13

WT, wild-type; NT, non-transgenic peanut coming from tissue culture; 3, 7, 11 and 13, four independent IPT-transgenic peanut plants. Oil content and fatty acid composition are in percentages (%). $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{20:0}$, arachidic acid; $C_{20:1}$, gadoleic acid; $C_{22:0}$, behenic acid; $C_{24:0}$, linoceic acid.
The SE is given. *significant at 5%; **significant at 1%; n = 5 seeds for each line.

vermiculite that was well watered (Premier Brands), germinated in a growth chamber under controlled conditions (25°C, 500 μmol photons $m^{-2} s^{-1}$, 16 h photoperiod) for 15 d and grown for another 15 d without irrigation. After that, half of the wild-type and transgenic plants were selected to receive 300 ml of water every 3 d (designated as optimal watering conditions), whereas the other half of the plants received 150 ml of water every 6 d (reduced irrigation conditions). Plants were grown for another 45 d. No water drained out of the pots in these treatments. After the above-mentioned reduced irrigation treatments were finished, biomass was determined by measuring fresh weight and dry weight. The fresh weight of each individual whole plant was measured immediately after harvest. Dry weight was measured after 48 h at 60°C in an air oven.

Molecular analysis of transgenic peanut plants

PCR and DNA blot analysis. Genomic DNA was isolated from fresh peanut leaves using the PowerPlant™ DNA Isolation Kit (Mo Bio Laboratories, Inc.). PCR was performed to screen putative transformants for the presence of the *SARK* promoter/IPT cassette with oligonucleotides pSARK-F1 and IPT-R1 (the 19 nucleotide primer pSARK-F1 is specific for the *SARK* promoter and the 20 nucleotide primer IPT-R1 is specific for *IPT*). The amplification reaction was carried out in a total volume of 25 μl containing 100 ng of purified genomic DNA as template, 1 U of GoTaq DNA polymerase (Promega Corporation) and 0.4 μM of each forward and reverse primer. Cycling conditions comprised an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 40 s and a final extension of 10 min at 72°C. The amplified products were electrophoresed on 1.2% agarose gels and visualized in the presence of ethidium bromide with a gel documentation system.

Aliquots of 10 μg of DNA were digested overnight with 20 U of *Hin*dIII, electrophoresed in a 0.8% agarose gel and transferred onto a Biotrans™ nylon membrane (MP Biomedicals, Inc.). The membrane was hybridized with a ^{32}P-labeled *IPT* cDNA fragment (50 ng) that was amplified by PCR using the oligonucleotides IPT-F2 and IPT-R2. The probe was made through random priming using a kit (DECA Prime™ Kit, Ambion Inc.). The conditions for hybridization and washing were described in Church and Gilbert (1984). The sequences of the oligonucleotides used are: pSARK-F1, 5′-GGTCATTGGCTTAGGGTTC-3′; IPT-R1, 5′-TCGTTCCTTTCAGTTCTTCC-3′; IPT-F2, 5′-CCAACTTGCACAGGAAAGAC-3′; and IPT-R25′-CTAATACATTCCGAACGGATGAC-3′.

RNA blot analysis. Total RNAs were isolated from the fifth leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Inc.). A 12 μg aliquot of total RNAs per sample was electrophoresed in 1% (w/v) formaldehyde–agarose gels, and transferred onto a Biotrans™ nylon membrane (MP Biomedicals, Inc.). RNA blot analysis was carried out following the protocol of Church and Gilbert (1984).

Gas exchange measurements

Gas exchange measurements were conducted on the third nodal leaf using a Licor LI-6400 infrared gas exchange analyzer (Licor, Inc.). Leaf-to-air vapor pressure deficit was set to ambient conditions, block temperature was 25°C, and the CO_2 concentration was set at 400 μmol mol^{-1}) and maintained for all measurements across pots. Irradiance was set to 1,500 μmol $m^{-2} s^{-1}$ using a light-emitting diode (Licor LI-6400-002). Leaves were first equilibrated in the chamber for at least 5 min prior to logging measurements of gas exchange. Field measurements of photosynthesis were taken under an irradiance of 2,000 μmol photons $m^{-2} s^{-1}$ using a light-emitting diode (Licor LI-6400-002)and 370 μmol mol^{-1} CO_2 in the leaf chamber of a Li-Cor 6400. Leaf temperature was maintained at 25°C. Gas exchange measurements were carried out in the middle of August, starting at 09:00 h and continuing until 12:00 h.

Field trial experiments

The wild-type controls and transgenic lines were planted at the Experimental Farm of the USDA-ARS Cropping Systems Research Laboratory in Lubbock, Texas, in the middle of May in both 2009 and 2010 (wild-type and three transgenic lines 3, 7 and 11 were used in 2009 and wild-type, a non-transgenic line from tissue culture, and the four transgenic lines 3, 7, 11 and 13 were used in 2010). In 2009, one treatment was tested: dryland with occasional flood irrigation. In 2010, two treatments were tested: reduced irrigation (19 mm per week) and high irrigation (38 mm per week). Seeds were sown into 2 m rows at a density of 20 seeds m^{-1} and a 100 cm row spacing in a 16 row block in 2009 and a 24 row block in 2010. Individual plants were randomly selected each time from *IPT*-containing peanut lines for gas exchange analysis, and other physiological data such as weight of peanut seeds and dry weight of above-ground biomass were collected at the end of experiments.

Peanut oil analysis

Oil content analysis. Time domain nuclear magnetic resonance (TD-NMR) experiments were carried out on a Bruker Minispec MQ10 NMR Analyzer with a tube of 25 mm diameter. The temperature was maintained at 40°C. Data were acquired using the Minispec software (Bruker), and the data oil program (Bruker). The weighed peanut samples (about 10 g) filled the tube for analysis. The 90° and 180° pulse lengths were 11.62 and 23.44 μs, respectively. For each run, 16 scans were collected with a recycle delay of 2.00 s. The gain was set to 60 dB. The TD-NMR instrument was calibrated by eight pure peanut oil calibration standards at the concentrations of 0, 10, 20, 30, 40, 50, 60 and 70%.

Fatty acid composition analysis. Peanut seeds were cut and powdered into hexane. The solvent was evaporated in nitrogen gas and the resulting fatty acids were converted to their methyl esters using boron trifluoride as a catalyst

in a methanol–toluene mixture at 95°C. The methyl esters were extracted into hexane and analyzed by HP 5890 gas chromatography (GC) with a flame ionization detector (FID) and a capillary column (DB-Wax, 30 m length, 0.53 mm i.d., 0.50 μm film thickness). Helium was used as the carrier gas at 30 p.s.i. A temperature program was used with an initial temperature of 200°C held for 1 min. The temperature was increased to 230°C at 3°C min^{-1}, then held for another 3 min at the final temperature. The injector was heated to 200°C and the split flow was 4 p.s.i. The detector temperature was 320°C. Fatty acids were identified by comparison with fatty acid methyl ester standards (68A) purchased from Nu-Chek Prep, Inc. Fatty acids were quantified by using the peak area percentage as a ratio to the total area of all methyl esters present.

Statistical analysis

Reduced irrigation experiments in a growth chamber were performed three times with 3–4 independent lines and 6–8 plants for each line each time. Means of one representative experiment are presented. The statistical significance of differences in the mean values of the examined parameters between the transgenic and wild-type plants was determined using the Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

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