Metabolite and mineral analyses of cotton near-isogenic lines introgressed with QTLs for productivity and drought-related traits

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Quantitative trait loci (QTLs) for yield and drought-related traits were exchanged via marker-assisted selection between elite cultivars of two cotton species, \textit{Gossypium barbadense} (GB) cv. F-177 and \textit{Gossypium hirsutum} (GH) cv. Siv’on. Three of the resultant near-isogenic lines (NILs), each introgressed with a different QTL region, expressed an advantage in osmotic adjustment (OA) and other drought-related traits relative to their recipient parents. These NILs and the parental genotypes were field-grown under well-watered and water-limited conditions, and characterized for their metabolic and mineral compositions. Comparisons were then made between (1) GB and GH genotypes, (2) the contrasting water regimes and (3) each NIL and its recipient parent. Hierarchical clustering analysis clearly distinguished between GB and GH genotypes based on either metabolite or mineral composition. Comparisons between well-watered and water-limited conditions in each of the genotypes showed differing trends in the various solutes. The greater concentrations of potassium, magnesium and calcium under water stress, when compared with well-watered conditions, may have enhanced OA or osmoprotection. All NILs exhibited significantly modified solute composition relative to their recipient parents. In particular, increased levels of alanine, aspartic acid, citric acid, malic acid, glycerol, myoinositol, threonic acid, potassium, magnesium and calcium were found under drought conditions in one or more of the NILs relative to their recipient parents. The increased values of these solutes could contribute to the superior capacity of these NILs to cope with drought.

Introduction

Drought, induced by soil and/or atmospheric water deficit, poses the most important environmental constraint to plant survival and crop productivity (Araus et al. 2002, Boyer 1982). With the anticipated environmental shift toward greater aridity and population growth, water is expected to become even more scarce in the near future (Chaves et al. 2003). Developing drought-resistant crop plants is, therefore, vital to meeting the increasing demand for agricultural products (Habash et al. 2007, Parry et al. 2005, Plucknett et al. 1987). This solution, however, requires a comprehensive understanding of plant drought-adaptive mechanisms at the physiological and genetic levels.

Abbreviations – GB, \textit{Gossypium barbadense}; GC–MS, gas chromatography–mass spectrometry; GH, \textit{Gossypium hirsutum}; HCA, hierarchical clustering analysis; NIL, near-isogenic line; OA, osmotic adjustment; OP, osmotic potential; QTL, quantitative trait locus; TCA, tricarboxylic acid.
A common response to drought stress in all kingdoms is the accumulation of minerals and compatible metabolites which are a part of normal metabolism (Bohnert and Sheveleva 1998). Plant species and cultivars differ greatly with respect to the types of metabolites accumulated, being various amino acids, sugars, polyols, quaternary amines and organic acids (reviewed by Zhang et al. 1999). Various functions have been suggested for the accumulated solutes. Osmotic adjustment (OA), the active accumulation of solutes in response to water deficit resulting in reduced osmotic potential (OP), contributes to improving water retention and cell turgor pressure as water deficit develops (Blum 1988, Morgan 1984, Turner and Jones 1980). Consequently, OA might contribute to sustaining physiological processes, such as stomatal opening, photosynthesis and expansion growth (Serraj and Sinclair 2002). Accumulated compatible solutes can replace water in certain biochemical reactions and can also associate with lipids or proteins and prevent membrane disintegration, the dissociation of protein complexes or the inactivation of enzymes (i.e. osmoprotection) (Bohnert and Jensen 1996). Metabolite profiling has been used to gain both diagnostic and mechanistic insights into plant responses and adaptations to a wide range of stresses, including water stress (Cramer et al. 2007, Mane et al. 2008, Rizhsky et al. 2004, Semel et al. 2007, Vasquez-Robinet et al. 2008), salt stress (Cramer et al. 2007, Gong et al. 2005, Johnson et al. 2003, Kim et al. 2007) and extreme temperatures (Kaplan et al. 2004; Rizhsky et al. 2004).

Cotton (Gossypium spp.; Malvaceae family) is the world’s leading fiber crop (http://www.fao.org) and among the most important oilseed crops. Gossypium barbadense and Gossypium hirsutum (hereafter GB and GH, respectively) are the two predominant cotton species, usually grown during the summer in arid and semiarid regions where water availability is often limited. Regardless of whether it is irrigated or not, cotton is often exposed to drought, which reduces both yield and lint quality (Pettigrew 2004).

In a previous study, quantitative trait loci (QTLs) for yield- and drought-related physiological traits – OP, carbon isotope ratio (an indicator of water-use efficiency) and leaf chlorophyll content, were exchanged via marker-assisted selection between elite cultivars of the two cotton species GB cv. F-177 and GH cv. Siv’on (Levi et al. 2009b). The resultant near-isogenic lines (NILs) rarely exhibited an advantage in yield under water-limited conditions. However, a considerable number of the NILs exhibited the physiological traits that had been targeted for introgression as well as remarkable modifications in non-targeted traits (Levi et al. 2009a, b). Several NILs introgressed with QTL regions conferring lower OP expressed an advantage in OA and other drought-related traits relative to their recipient parents (Levi et al. 2009b). Three of these NILs, each introgressed with a different QTL region, were targeted in this study.

The metabolic and mineral compositions of the selected NILs and their recipient parents were characterized in order to (1) compare between GB and GH cotton genotypes, (2) characterize the effects of contrasting water regimes and (3) compare each NIL with its recipient parent.

Materials and methods

Development of NILs

Seven genomic regions containing QTLs associated with productivity and drought-related traits, OP, carbon isotope ratio and leaf chlorophyll content, were selected for the development of NILs (Levi et al. 2009b). A marker-assisted backcross program was conducted for the introgression of targeted regions using GB cv. F-177 and GH cv. Siv’on as donor and recipient genotypes. The procedure used well-established protocols for genomic DNA extraction (Paterson et al. 1993) and RFLP marker analysis (Reinisch et al. 1994).

Donor genotypes were drawn from the original F3 mapping population, genotyped with 279 DNA markers. Plants containing the favorable allele at the targeted region, with a minimum of chromatin from the donor species (an average of about 40%) were backcrossed three times to the recipient parent to produce BC3F1 progenies. In BC3F1 as well as in BC3F2 progenies, 25 plants per target region were genotyped with the appropriate markers, flanking and within the target regions, to identify plants heterozygous at the target markers. For each target region, about 10 BC3F1 plants were self fertilized to produce 40 BC3F2 progenies, which were genotyped to identify plants homozygous at the target markers. Subsequently, BC3F3 progenies genotyped with a total of 105 microsatellite markers revealed an average 6.6% of the donor genome (including the target region).

Three of the resultant NILs were used in this study

1. NIL 1-1: GB as the recipient parent introgressed with a genomic region (Linkage group A02 flanked by pAR792 and pGH232a markers) from GH as the donor line.
2. NIL 2-2: GB as the recipient parent introgressed with a genomic region (Chromosome 06 flanked by pAR936 and G1099 markers) from GH as the donor line.
3. NIL 3-2: GH as the recipient parent introgressed with a genomic region (Chromosome 25 flanked
by pAR969 and pAR839 markers) from GB as the donor line.

Plant growth conditions and sampling

The NILs and their parental genotypes were field-grown under two water treatments: well-watered control (653 mm, consistent with commercial cotton practices) and water-limited (357 mm). A split-plot factorial (line × irrigation regime) block design with six replicates was employed, with irrigation treatment in main plots and genotypes in subplots. The trial was sown on April 9, 2006 at the experimental farm of the Hebrew University of Jerusalem in Rehovot located in the coastal plain of Israel (31°54′N, 34°47′E). Complete details of the experimental conditions and procedures have been described previously (Levi et al. 2009b). Samples for metabolite and mineral analyses were collected during mid-flowering stage, a time at which difference in turgid leaf OP between well-watered and water-limited plants was approximately 0.4 MPa. Each biological replicate consisted of a pool of 18 leaf discs, sampled from the youngest fully expanded leaf and the two leaves below it from two adjacent plants per plot (three discs from each leaf). Samples were collected from the field, immediately frozen in liquid Nitrogen and kept at −80°C until analyzed. A total of five biological replicates (field plots) were analyzed for metabolic profiles and six replicates for minerals.

Metabolite extraction and derivatization

The extraction protocol for metabolites was modified from Roessner-Tunali et al. (2003). Briefly, frozen leaf tissue was lyophilized, powdered and extracted in 500 ml of 80% (v/v) aqueous ethanol. Ribitol (30 μl of 0.2 mg ml$^{-1}$ water) was added as an internal standard prior to incubation. The mixture was extracted in an incubator-shaker (15 min at 70°C, 200 rpm). The extract was vigorously mixed with 600 μl water and 300 μl chloroform, and subsequently centrifuged for 5 min at 20 800 g. Aliquots of the methanol/water supernatant (80 μl) were lyophilized. The dry residue was modified for gas chromatography–mass spectrometry (GC–MS) analysis according to Schauer et al. (2005). Residues were re-dissolved and derivatized for 2 h at 37°C in 40 μl of 20 mg ml$^{-1}$ methoxyamine hydrochloride in pyridine followed by a 30 min treatment with 70 μl of N-methyl-N-[trimethylsilyl] trifluoroaceticamide at 37°C. A mixture of retention time standards [7 μl of 0.029% (v/v) n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane and n-hexatriacontane dissolved in pyridine] was added before trimethylsilylation. Sample volumes of 1 μl were then injected into the GC column in a splitless model.

GC–MS analysis and chromatogram evaluation

The GC–MS system was run as previously described (Schauer et al. 2005) with some modifications. It was composed of a Pal autosampler (CTC Analytic, Zwingen, Switzerland), a TRACE GC 2000 gas chromatograph, and a TRACE DSQ quadrupole mass spectrometer (Thermo Finnigan, Hemel Hempstead, UK). GC was performed on a 30 m Rtx-5Sil MS column with 0.25-μm film thickness (Thermo Finnigan). The injection temperature was set at 297°C, the interface at 280°C and the ion source adjusted to 200°C. Helium was used as the carrier gas at a flow rate of 1 ml min$^{-1}$. The analysis was performed under the following temperature program: 5 min of isothermal heating at 70°C, followed by a 5°C min$^{-1}$ oven temperature ramp to 350°C and a final 5 min heating at 330°C. Mass spectra were recorded at 2 scan s$^{-1}$ with a scanning range of 40–600 m/z. Both chromatograms and mass spectra were evaluated using the xcalibur VI 3 program (Thermo Finnigan). A retention time and MS library for automatic peak quantification of metabolite derivatives was implemented within the NIST 2.0 method format. Substances were identified by comparison with authentic standards, as described in Roessner-Tunali et al. (2003). The levels of the compounds were calculated as the relative response ratio of peak areas of different compounds related to the peak area of ribitol (which served as an internal standard), and normalized with respect to the dry weight of the sample.

Mineral extraction and analysis

Leaf concentrations of mineral nutrients (i.e. calcium, magnesium, potassium, sodium and sulfur) were measured on the same leaf samples used for metabolomic profiling. Samples were digested in a closed microwave system and concentrations of minerals were determined by inductively coupled plasma–optical emission spectrometry (ICP–OES; Vista-Pro Axial, Varian Pty Ltd., Mulgrave, Australia). Measurements of minerals were checked using the certified values of the related minerals in a reference leaf sample received from the National Institute of Standards and Technology (NIST; Gaithersburg, MD).

Statistical analysis

The JMP® 7.0 statistical package (SAS Institute, Cary, NC) was used for statistical analysis. Hierarchical
clustering analysis (HCA) was conducted using the Ward method. A two-way split-plot model was employed for ANOVA with genotype and irrigation as fixed effects, and block and block × irrigation as random effects. Student’s t-test was used to compare between the two recipient parents in each of the irrigation treatments, irrigation treatments for each of the genotypes and each NIL and its recipient parent under each irrigation treatment.

Results and discussion

Hierarchical clustering analysis

HCAs based on either 27 metabolites (Fig. 1A) or five minerals (Fig. 1B) revealed a most pronounced difference between GB and GH genotypes, and in most cases a difference between irrigation treatments as well. In addition, NIL 1-1 was subdivided from the other GB genotypes under both irrigation treatments. Accordingly, comparative analyses of metabolites and minerals were conducted at three different levels:

(1) between GB and GH cotton, represented by F-177 and Siv’on, respectively; (2) between well-watered and water-limited treatments in each of the tested genotypes and (3) between each NIL and its recipient parent under each irrigation treatment.

Differences between GB and GH cotton

The recipient parents, Siv’on and F-177, differed from one another in 12 or 11 metabolites out of 27 under the well-watered and water-limited treatments, respectively. These differences included amino acids, organic acids and polyols (Table 1). Siv’on exhibited higher levels of the metabolites that differed between these genotypes in 50% of the cases in the well-watered treatment (ascorbic, citric, glyceric, malic, quinic acids and putrescine) and in 73% of the cases in the water-limited treatment (alanine, caffeic, dehydroascorbic, glyceric, malic, quinic, shikimic acids and putrescine). The recipient parents also differed in four of the five mineral nutrients (calcium, magnesium, sodium and sulfur), similarly in both

Fig. 1. HCA of GB and GH genotypes under well-watered and water-limited conditions. (A) HCA based on metabolite profiling. Five replications of each genotype × irrigation combination were averaged for each metabolite. (B) HCA based on mineral concentrations. Six replications of each genotype × irrigation combination were averaged for each metabolite.
Table 1. Metabolite profiling of Gossypium barbadense and Gossypium hirsutum genotypes grown under well-watered and water-limited treatments. Data were normalized with respect to the mean value for the well-watered F-177. Values are the means of five replicates. Statistical differences between means of F-177 and Siv’ on within each irrigation treatment (∗, ++ and +++), between each NIL and its recipient parent within each irrigation treatment (∗, ** and ***), and between irrigation treatments within each of the genotypes (∼, ∼∼ and ∼∼∼) are at P ≤ 0.05, 0.01 and 0.001, respectively.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>G. barbadense genotypes</th>
<th>G. hirsutum genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Well-watered</td>
<td>Water-limited</td>
</tr>
<tr>
<td></td>
<td>F-177</td>
<td>NIL 1-1</td>
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<td>Aspartic acid</td>
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<td>Glycine</td>
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While crossability and phylogenetic analyses of both nuclear and cytoplasmic DNA sequences indicate that GB and GH are closely related (Small et al. 1998, 1999), they are quite different in morphophysiological characteristics such as leaf morphology, flower initiation, ripening time, lint yield and quality. Siv’ on (GH) exhibits higher water-use efficiency than F-177 (GB) under field conditions (Saranga et al. 1998), and is better adapted to drought. This study showed that these genotypes clearly differed from one another in their metabolite (Fig. 1A) and mineral (Fig. 1B) compositions, under both control and drought conditions (Tables 1 and 2). The accumulation of solutes under drought has been suggested as an adaptive mechanism for drought and salt tolerance to maintain a better water regime at the cellular level (reviewed by Serraj and Sinclair 2002). Under drought conditions, most metabolites and minerals that differed between the recipient parents exhibited greater values in Siv’ on relative to F-177, which could contribute to the former’s greater drought resistance (Saranga et al. 1998, 2004). These differences set the background for further comparisons between each NIL and its recipient parent.

**Effect of drought on solute composition**

Comparisons between well-watered and water-limited conditions in each of the genotypes showed differing trends for the various metabolites (Table 1). The expression of drought tolerance depends on the energy status of the cells in which the appropriate responses
are being induced (Hare et al. 1998). Many tissues of stressed plants are likely to have a higher demand for rapidly metabolizable carbohydrates. This must be satisfied despite a possible decrease in carbon fixation and increased diversion of carbon from growth or storage to osmolyte synthesis. The reduced levels of soluble sugars (and other metabolites) under drought stress in most genotypes in this study (Table 1), as well as in previous reports (Cramer et al. 2007, Ghasempour et al. 1998), may reflect the metabolic cost of drought responses. On the other hand, metabolites that increase under water stress may contribute to drought adaptation and therefore warrant special attention.

All GB genotypes showed higher levels of saccharic acid under the water-limited treatment than under the well-watered control treatment. NIL 2-2 (GB) exhibited higher levels of aspartic acid, glyceric acid and myoinositol in response to drought. GH genotypes showed higher levels of serine, malic acid and threonic acid under the water-limited treatment. In addition, Siv’on exhibited higher levels of glyceric acid, shikimic acid and putrescine.

Among the mineral nutrients tested in this study, potassium showed significant increases in all GB genotypes (approximately 60%) and also in the GH genotype Siv’on (34%) in response to water stress (Table 2), suggesting a major role of potassium in OA. This pronounced increase in leaf potassium concentrations under water stress appears to be very specific, because other mineral nutrients measured exhibited smaller changes or even decreased (Table 2). The contribution of potassium to plants’ adaptation to various stress conditions (Cakmak 2005, Chen et al. 2007, Rascio et al. 2001), and particularly to OA (Brini et al. 2007, Kusaka et al. 2005, Morgan 1992, Rascio et al. 1994), is well documented. In addition, the particularly high potassium concentration induced by drought in the GB genotypes suggests stimulated root uptake and/or root-to-shoot transport under water stress. A further examination of potassium uptake and transport capacity in those genotypes under various drought stress treatments would therefore be of interest.

Magnesium was also accumulated to higher amounts under water stress vs the control treatment in two GB genotypes, F-177 and NIL 1-2, and the GH recipient parent, Siv’on (Table 2). Magnesium has been suggested to alleviate the detrimental effects of various stress factors such as drought (Cakmak and Kirkby 2008). In addition, it is a component of the chlorophyll molecules (Taiz and Zeiger 2002), hence the increased concentration of magnesium under drought in several genotypes (Table 2) may relate to their increased leaf chlorophyll content under drought conditions (Levi et al. 2009b).

Finally GH genotypes exhibited higher levels of calcium under water-limited conditions than under the well-watered treatment. A protective role has been attributed to high levels of calcium in stabilizing cell membranes (Palta 1996) and in inducing increased antioxidant enzyme activities under abiotic stress (Gong et al. 1997; Nayyar and Kaushal 2002).

### Solute composition in NILs relative to their recipient parents

Each of the NILs tested in this study have been previously shown to exhibit improved drought-adaptive responses as compared with their recipient parent (Levi et al. 2009a, b). The NILs usually exhibited greater OA under drought conditions than their recipient parents. In addition, NIL 1-1 and its sister lines (NIL 1-2 and NIL 1-4, with the same introgression and recipient parent) have been characterized by smaller leaf size (Levi et al. 2009b) and NIL 1-4 shows stable photosynthesis across a range of leaf water potentials and greater assimilation rate under drought than F-177 (Levi et al. 2009a). NIL 2-2 exhibited greater pubescence (unpublished data) and carbon isotope ratio than F-177. NIL 3-2 has been
characterized by higher mesophyll conductance than Siv’on under drought conditions. This discussion will focus on metabolites that were found at higher levels in the NILs than in their recipient parents, assuming a contribution to drought adaptation.

Under the water-limited treatment, NIL 1-1 differed from F-177 in seven metabolites, with three (aspartic, citric and malic acids) showing greater levels in NIL 1-1 (Table 1). The most dramatic changes occurred with the citric and malic acids which, in NIL 1-1, showed over twofold the levels in F-177 under drought (Fig. 2). In previous studies, citric acid has been shown to accumulate (over twofold) under drought in leaves of two GH strains characterized by enhanced performance under field water stress, whereas two other strains which were characterized by poor performance, accumulated lower levels of citric acid (Timpa et al. 1986). Higher levels of citric acid in response to drought have also been found in tomato (Semel et al. 2007), potato (Mane et al. 2008) and Arabidopsis (Rizhsky et al. 2004), and in response to extreme temperatures in Arabidopsis (Kaplan et al. 2004). Both malic and citric acids are intermediates of the tricarboxylic acid (TCA) cycle. In addition, α-ketoglutaric and succinic acids, which are also intermediates of the TCA cycle, were found at higher levels in NIL 1-1 than in F-177 (under the water-limited treatment not significant for α-ketoglutaric acid and nearly significant for succinic acid, P = 0.06). These findings suggest the involvement of TCA intermediate metabolites in the superior drought adaptation of NIL 1-1 relative to in F-177.

NIL 2-2 differed from F-177 under water limitation in five metabolites, four of which (alanine, aspartic acid, glyceric acid and glycerol) exhibited 40–60% higher levels in NIL 2-2 (Table 1). Glycerol has been suggested to function as either an osmolyte, contributing to the maintenance of water balance, or as an osmoprotectant, allowing the operation of many cellular processes under osmotic stress (Shen et al. 1999). The accumulation of

![Fig. 2](image-url)
glycerol in response to extreme temperatures has been reported in Arabidopsis (Kaplan et al. 2004). High levels of alanine (NIL 2-2) and aspartic acid (both NIL 1-1 and NIL 2-2) in response to drought conditions have been reported in tomato (Semel et al. 2007), Brassica napus (Good and Zaplachinski 1994) and potato (Mane et al. 2008). However, in B. napus, increased levels of amino acids, including alanine and aspartic acid, were accompanied by a reduction in overall protein synthesis, which may reflect a metabolic cost of metabolite accumulation (Serraj and Sinclair 2002). Under well-watered conditions, NIL 2-2 and F-177 exhibited similar levels of aspartic and glyceric acids; however, under water-limited conditions, the level of these metabolites was doubled in NIL 2-2 while in F-177, they exhibited a non-significant increase. Hence, aspartic and glyceric acids may contribute to the higher OA previously observed in NIL 2-2 (Levi et al. 2009b).

NIL 3-2 differed from Siv’on in seven metabolites under each treatment; however, under the water-limited treatment, it exhibited higher levels of myoinositol and threonic acid (Table 1). Interestingly, in NIL 1-1, with the GH genomic region introgressed into GB, levels of myoinositol were lower than in the recipient parent, F-177, under both treatments. Myoinositol has been postulated to act as an osmolyte or osmoprotectant, or serve as a storage of carbohydrate under stress (Klages et al. 1999). Increased levels of myoinositol in response to heat–shock stress have been reported in Arabidopsis (Kaplan et al. 2004) and in response to salt stress in several other plants (refer Klages et al. 1999 and references therein). In Mesembryanthemum crystallinum, it has been shown to play a central role in metabolic responses leading to salt tolerance (Nelson et al. 1998). However, its accumulation in response to salt stress in tobacco and Actinidia species was not associated with salt tolerance (Klages et al. 1999, Sheveleva et al. 1997).

All three NILs studied, exhibited either similar or greater mineral concentrations than their recipient parents (Table 2). Three mineral nutrients, potassium, magnesium and calcium, which increased in response to water stress, are of major interest. Relative to their recipient parents, NIL 1-1 exhibited a greater concentration of potassium, magnesium and calcium under both treatments (Fig. 2). NIL 2-2 exhibited a higher concentration of potassium under drought and NIL 3-2 exhibited a higher concentration of potassium under the well-watered treatment (Table 2). The roles of these minerals in drought adaptation were discussed above. The increased levels of these minerals in the tested NILs may, therefore, contribute to their drought adaptation relative to their recipient parents, as shown previously (Levi et al. 2009a, b).

Conclusions

This study provides the first comparative analysis of metabolite and mineral compositions in leaves between two cultivars of the predominant cotton species GB and GH. The clear distinction between these genotypes in both metabolite and mineral compositions corresponds with differences at the whole-plant phenotype level. Another unique aspect of this study arises from the comparison between two recipient parents and their derivative NILs introgressed with QTLs conferring greater OA from GH to GB (NILs 1-1 and 2-2) and vice versa (NIL 3-2). These plant materials exhibited specific changes in metabolic and mineral composition (e.g. potassium and magnesium) in otherwise nearly identical genetic backgrounds.

The mechanisms underlying the changes in metabolite and mineral concentrations presented here have yet to be clarified. Comparative analyses at the mineral, metabolic and genomic levels may help to delineate the solute and gene networks underlying cotton adaptation to drought and other abiotic stresses. Furthermore, burgeoning tools for cotton genomics (Paterson et al. 2010, Reinisch et al. 1994, Rong et al. 2004, 2005, Udall et al. 2006) may clarify the nature of the target QTLs and expedite their cloning. Therefore, the availability of such tools and the materials used in this study provide a unique opportunity for further investigation of crop adaptation to arid conditions.

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Physiol. Plant. 141, 2011

275