Transcriptomic analysis comparing stay-green and senescent Sorghum bicolor lines identifies a role for proline biosynthesis in the stay-green trait

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Abstract

Sorghum bicolor is an important cereal crop grown on the arid and semi-arid regions of >98 different countries. These regions are such that this crop is often subjected to low water conditions, which can compromise yields. Stay-green sorghum plants are able to retain green leaf area for longer under drought conditions and as such have higher yields than their senescent counterparts. However, the molecular and physiological basis of this drought tolerance is yet to be fully understood. Here, a transcriptomic approach was used to compare gene expression between stay-green (B35) and senescent (R16) sorghum varieties. Ontological analysis of the differentially expressed transcripts identified an enrichment of genes involved with the ‘response to osmotic stress’ Gene Ontology (GO) category. In particular, delta1-pyrroline-5-carboxylate synthase 2 (P5CS2) was highly expressed in the stay-green line compared with the senescent line, and this high expression was correlated with higher proline levels. Comparisons of the differentially expressed genes with those that lie in known stay-green qualitative trait loci (QTLs) revealed that P5CS2 lies within the Stg1 QTL. Polymorphisms in known cis-elements were identified in the putative promoter region of P5CS2 and these could be responsible for the differences in the expression of this gene. This study provides greater insight into the stay-green trait in sorghum. This will be greatly beneficial not only to improve our understanding of drought tolerance mechanisms in sorghum, but also to facilitate the improvement of future sorghum cultivars by marker-assisted selection (MAS).

Key words: Drought, microarray, proline, sorghum, stay-green.
Consequently, drought stress is thought to be the biggest cause of crop yield reduction, particularly in the arid and semi-arid tropics (Boyer, 1982; Passioura, 2007). There is therefore an urgent requirement to improve our understanding of how crops are able to tolerate conditions of low water availability in order to help limit the problems associated with drought in the future.

The mechanisms of drought tolerance are complex, and the response of the plant depends upon the growth stage and the severity of the stress encountered (Rosenow et al., 1983). With regard to plant morphology, an increased root depth and a thick waxy leaf cuticle can help plants to extract and retain more water, respectively. Physiological adaptations can allow for the control of water loss by transpiration via the stomata, and biochemical adaptations can act both to delay and to reduce the effects of the drought stress (Chaves et al., 2003). One such biochemical mechanism is the accumulation of compatible solutes including proline, glycine betaine, and trehalose. These are not only known to act via osmotic adjustment mechanisms to help maintain cell turgor but are also thought to be able to counteract the harmful effects of reactive oxygen species (ROS) (Ashraf and Foolad, 2007).

Sorghum bicolor is an important C₄ grain crop that is grown in the arid and semi-arid tropics and is known to be particularly well adapted to conditions of low water availability. It is the fifth most important cereal crop grown worldwide, based on yield, and is an important source of food, feed, fibre, and fuel (Kholova et al., 2013). Whilst numerous studies have investigated the physiological mechanisms underlying drought tolerance in sorghum, relatively little is known about the adaptations at the biochemical and molecular level. In the field, agricultural traits conferring drought tolerance have been identified, including the stay-green trait (Rosenow et al., 1983; Sanchez et al., 2002; Thomas and Ougham, 2014). Plants possessing the stay-green trait are able to maintain green photosynthetic leaf area for longer under drought stress conditions at the post-flowering stage and, as a result, produce higher grain yields than their drought-sensitive counterparts (Rosenow et al., 1983; Borrell et al., 2000; Harris et al., 2007). Several sorghum genotypes have been identified that exhibit the stay-green trait, including B35, SC56, and E36-1 (Rosenow et al., 1983; Kebede et al., 2001; Haussmann et al., 2002; Sanchez et al., 2002). Of these, B35 is the best characterized, with a number of physiological studies being carried out on this variety or its derivatives (Crasta et al., 1999; Xu et al., 2000a; Kassahun et al., 2010; Vadez et al., 2011).

Previous studies investigating the stay-green trait in sorghum have identified differences in chlorophyll content, transpiration, relative water content (RWC), and nitrogen status when comparing stay-green and senescent lines (Borrell and Hammer, 2000; Thomas and Howarth, 2000; Xu et al., 2000a; Harris et al., 2007; Vadez et al., 2011). Other studies have identified differences in tillering and leaf size which could impact upon pre-flowering water usage (Borrell et al., 2014a, b). As a result, it is thought that the increase in grain yield in the stay-green varieties following stress at the post-flowering stage can be attributed to the emergent consequence of genes acting at the pre-flowering stage (Borrell et al., 2014a).

Mapping studies based on a number of crosses, largely based on the B35 stay-green line, have also been able to identify four quantitative trait loci (QTLs; Stg1–Stg4) for the trait that are consistent across different backgrounds (Crasta et al., 1999; Subudhi et al., 2000; Xu et al., 2000b; Sanchez et al., 2002; Harris et al., 2007). These have been introgressed into the high yielding but senescent R16 background (Kassahun et al., 2010; Vadez et al., 2011). This trait is, however, undoubtedly complex and, despite these advancements, the physiological and molecular basis of this trait remains unclear. Such an understanding would be greatly beneficial not only to improve our understanding of drought tolerance mechanisms in sorghum but also to facilitate the improvement of future sorghum cultivars by marker-assisted selection (MAS).

Transcriptomic analyses, including microarrays, are a valuable way in which mechanistic insights into biological phenomena can be obtained. For example, the comparison of gene expression in different samples can provide insight into the actual biological processes that are perturbed following a specific treatment or between different genotypes. In sorghum, a number of transcriptomic experiments have been carried out recently and have led to the identification of many stress-related transcripts (Zhu-Salzman et al., 2004; Buchanan et al., 2005; Salzman et al., 2005; Park et al., 2006; Dugas et al., 2011; Johnson et al., 2014). The recent release of the sorghum genome sequence (Paterson et al., 2009) and the development of a metabolic pathways database, SorghumCyc (http://pathway.gramene.org/gramene/sorghumCyc.shtml) have greatly facilitated these studies. Microarray analysis could therefore be a powerful approach for elucidating some of the molecular and biochemical pathways involved in conferring the stay-green trait in sorghum.

Here, using microarray analysis, gene expression differences between a stay-green and senescent line are described. Ontological analysis of the differentially expressed genes suggested a potential role for a number of processes in the stay-green trait and in particular a role for proline biosynthesis. This was validated biochemically, and a putative mechanism to explain the higher proline levels in the B35 stay-green line is presented. The data suggest that at least part of the stay-green phenotype of the B35 line is due to increased free proline levels.

Materials and methods

Plant growth conditions and sampling of tissue

Seeds of sorghum (Sorghum bicolor L. Moench.) R16 and B35 (BT×642) varieties were soaked in water overnight and surface sown singly onto rehydrated 44 mm Jiffy peat pellets (LBS Horticulture Ltd, Lancashire, UK). Seedlings were grown in a glasshouse at 28 °C day, 23 °C night, 12 h photoperiod, and ~1000 μm mol m⁻² s⁻¹. At 30 days after sowing (DAS), the seedlings were transferred to 8 inch pots containing New Horizon Organic and Peat Free Compost (William Sinclair Horticulture Ltd, Lincolnshire, UK). From this point, the photosynthetic efficiency of leaves 2 and 4 was monitored using a portable photosystem efficiency analyser (PEA) machine (Hansatech, Norfolk, UK). A sample from leaf 10 was taken when the average photosynthetic efficiency of leaves 2 and 4 first started to differ between the B35 and R16 varieties, as indicated by a reduced ratio of variable fluorescence (Fv) to maximal fluorescence (Fm) in R16. This occurred
at ~45 DAS (Supplementary Fig. S1 available at JXB online). At this stage the plants were at the booting stage and had 10 leaves. Leaf 10 was sampled and the tissue was pooled from six plants of each variety. Plants were maintained under well-watered conditions throughout. Experiments were carried out in triplicate, with samples taken on different occasions, to give three biological replicates. Samples were taken at the same time of day for each biological replicate to reduce variation due to circadian/diurnal factors. Tissues samples were harvested at ~45 DAS (Supplementary Fig. S1 available at JXB online).

Microarray design, and cRNA synthesis and labelling
Total RNA was isolated using the Qiagen miRNeasy Mini Kit with QIAshredder columns for tissue homogenization (Qiagen, Sussex, UK). The integrity of the RNA was confirmed using an Agilent 2100 bioanlyser (Palo Alto, CA, USA) and the RNA 6000 Nano Kit (Agilent). Custom expression microarrays (4×44K format) for sorghum were designed and submitted for manufacturing using the Agilent Technologies eArray web-based application (https://earray.chem.agilent.com/earray/), as described previously (Johnson et al., 2014). All products were obtained from Agilent Technologies UK Ltd (Wokingham, Berkshire, UK) and used according to the Agilent ‘One Colour Low Input Quick Amp Microarray Based gene expression’ protocol, as described previously (Johnson et al., 2014). The labelled cRNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol and quantified using a UV-VIS spectrophotometer. The Agilent Hybridization Kit (Cat. no. 5188–5242) was used according to the manufacturer’s instructions, again as described previously (Johnson et al., 2014). The accession number for this microarray is GPL17335.

Bioinformatic analysis
The Agilent Feature Extraction Software (v10.7) was used to extract data from scanned microarray images. The extracted data were analysed using GeneSpring GX 11 software (Agilent Technologies). Controls, spots of poor quality (not detected), and gene probes which were not present in all three repetitions in either the control or treatment samples were excluded from the analysis. This yielded ~21 000 probes for the B35 versus R16 comparison. From these 21 000 genes, those with an average fold change (FC) of ≥2.0 and a P-value of ≤0.05 (moderated t-test with Benjamini–Hochberg correction) were selected for further analysis. Singular enrichment analysis (SEA) of Gene Ontology (GO) terms was determined using Agrigo (http://bioinfo.cau.edu.cn/agriGO/) (Du et al., 2010). Hierarchical clustering of normalized gene expression was carried out on conditions and entities using GeneSpring default settings. The SorghumCyc metabolic pathways database (http://pathway.gramine.org/gramine/sorghumcyc.shtml) was used to identify sorghum genes involved in particular biosynthetic pathways.

Real-time qPCR
Quantitative PCR (qPCR) validation of the microarray data was carried out using Fluidigm 96 Dynamic arrays (Fluidigm, San Francisco, CA, USA). Three additional biological replicates were set up and sampled as described above. Assays were run in triplicate to give three technical replicates of each biological replicate. The set-up was performed in accordance with the ‘Fluidigm® 96.96 Real-Time PCR Workflow’ (PN68000088) (http://fgl.salk.edu/BioMark/pdf/96.96%20Real-Time%20PCR%20Workflow%20Quick%20Reference%20rev%20C1.pdf). Total RNA (1 μg) was used as input in a 20 μl reverse transcription reaction. The SuperScript III First-Strand Synthesis SuperMix Kit (Applied Biosystems, Foster City, CA, USA, Cat. no. 11752-050) was used for first-strand cDNA synthesis, and the TaqMan PreAmp Master Mix (Applied Biosystems) was used for pre-amplification of the cDNA. Custom-designed Custom TaqMan® Gene Expression Assays (Applied Biosystems) were used for amplification of the cDNA, and data were collected using Fluidigm Real-Time PCR analysis Software v3.0.2 (see Supplementary Table S1 at JXB online for a full list of probes). Relative quantification was accomplished using the comparative Ct method (ΔΔCt method) (Livak and Schmittgen, 2001). Sb04g028990.1 was used as an endogenous control due to its unchanged expression following various stress treatments in previous microarray analyses (Johnson et al., 2014). qPCR of the P5CS transcripts was carried out using an AB 7300 real-time PCR system (Applied Biosystems) and GoTag qPCR master mix (Promega, Madison, WI, USA) as described previously (Moffat et al., 2012). The oligo sequences used (sequence direction 5′ to 3′) were: Sb03g039820.1 Fwd, TCACCGATGAAACGCAA; Sb03g039820.1 Rev, CCTCAACATCGTCCATTT; Sb09g02230.1 Fwd, GGGTCTTTAGCAATCCGAG; Sb09g02230.1 Rev, AAGTTTTCACCCACGT TG; Sb09g022310.1 Fwd, ATTCAGCTTCATCACCTGCT; and Sb09g022310.1 Rev, CATCATCAAGTGGGCCAGT.

Osmotic stress and proline quantification
Osmotic stress was administered at 14 DAS by application of 10% polyethylene glycol (PEG) to peat plugs, as described previously (Turkan et al., 2005). Leaf samples were taken following 3 d of water withholding and used both for RNA extraction for qPCR and for proline quantification. Proline levels were quantified using ultra-high performance liquid chromatography [UPLC; Waters Acquity H-Class UPLC® system with fluorescence (FLR) and photodiode array (PDA) detectors; Waters, Wilmslow, UK]. Leaf tissue was ground in liquid nitrogen and lyophilized overnight. Lyophilized tissue (0.04 g) was extracted in 1.5 ml of 0.1 N HCl by grinding and then centrifugation at 17 000 g for 20 min at 4 °C. The extracts were then sequentially derivatized with OPA (o-phthalaldialdehyde) reagent and FMOC (fluorenlymethylxylocarbonyl) (Sigma). OPA reagent consists of 260 mM N-isobutyl-l-cysteine (IBLC) (Sigma) and 170 mM OPA (Sigma) in 1 M potassium borate buffer (pH 10.4). The following reactions were set up in an HPLC vial: 10 μl of sample, 10 μl of OPA reagent, 20 μl of FMOC (5 mM in acetonitrile), and 60 μl of 100% methanol. Separations were performed on a Cortecs C18, 100 mm×2.1 mm, 1.6 μm column (Waters), and elution was achieved at 40 °C. Mobile phase A was made up of 20 mM sodium acetate, pH 6.0. Mobile phase B was made up of acetonitrile/methanol/water in a 45:45:10 (v/v/v) ratio. A flow rate of 400 μl min−1 was used. Automated HPLC injection added 3 μl of the sample for analysis, and samples were run for 20 min. For OPA detection, the excitation and emission wavelengths were 340 nm and 455 nm, respectively. For FMOC detection, the excitation and emission wavelengths were 266 nm and 305 nm, respectively. Quantification was made with reference to a proline standard that was derivatized and run with each sample set.

Stay-green QTLs and SNP identification
Genes within known QTLs for stay-green were identified using the Comparative Saccharinae Genome Resource (CSGR) (http://helos.pgi.mpg.de/QT2/) (Zhang et al., 2013). In order to compare the upstream promoter sequence, gDNA was extracted from the R16 and B35 varieties using the Dellaporta method (Dellaporta et al., 1983). PCR was carried out using BioTaq Polymerase (BIO-21040) and the following primers (sequence direction 5′ to 3′): −500Fwd, TTGTGGTCGTGTGGCACGT; −500 Rev, CGGGGGGGAATACTGGTGGGATC. Sequencing was performed at University of Durham on April 7, 2016.

Results
Microarray analysis identified genes differentially expressed in a stay-green (B35) versus a senescent line (R16) at the pre-flowering stage
In an attempt to better understand the molecular processes that are associated with the stay-green trait, microarray
analysis was carried out to capture gene expression differences between a stay-green and senescent line. Tissue samples were taken at ~45 DAS under well-watered conditions. At this time point there were higher levels of chlorophyll in B35 compared with R16, as indicated by measurements of chlorophyll fluorescence (see Supplementary Fig. S1 at JXB online); therefore, at least one known element of the trait was manifesting at this time point (Thomas and Howarth, 2000). Samples were harvested on three separate occasions to provide three biological replicates.

RNA was hybridized to custom-designed microarray chips containing 28 585 gene probes, as used previously (Johnson et al., 2014). As shown in Fig. 1, 1038 genes were expressed to higher levels (FC>2, \(P<0.05\)) in B35 compared with R16, and 998 genes were expressed to lower levels (Supplementary Tables S2, S3 at JXB online). These gene expression changes constitute 3.6% and 3.4% of total genes on the chip for the genes expressed to higher and lower levels, respectively. Differentially expressed genes identified in the microarray were validated using qPCR on an additional three biological replicates. Probes were designed to 87 genes and, of these, 83% showed a fold change in the same direction in both the qPCR and microarray analysis (see Fig. 2; Supplementary Table S4). The qPCR largely confirmed the results obtained by the microarray analysis, confirming the accuracy of the high-density microarrays and the robustness of the experimental system.

Ontological analysis of differentially expressed genes identified enriched biological processes in the stay-green B35 line

In order to identify the biological processes and molecular functions that are enriched within the differentially expressed gene sets, GO analysis was carried out. The AgriGO gene ontology tool (http://bioinfo.cau.edu.cn/agriGO/) was used to group genes into broad functional categories based on their GO annotations (Du et al., 2010). Singular enrichment analysis (SEA) was then carried out to identify particular GO categories that were significantly enriched (\(P<0.05\)) within the genes expressed at higher levels in B35 (Table 1). Enriched GO categories include processes such as ‘post-embryonic morphogenesis’ and ‘anatomical structure homeostasis’. Other enriched GO categories include ‘cell redox homeostasis’ and ‘cellular amino acid metabolic activity’. Notably, processes that are known to be associated with the plant response to low water availability were also enriched, including ‘response to osmotic stress’ and ‘water transport’. In order to determine whether genes associated with other known stress stimuli were differentially expressed, the percentage of genes associated with these stimuli was compared with the percentage of genes in the genome that are associated with that same stimulus. The ‘response to osmotic stress’ category was found to be the only strongly enriched stimulus, with nearly 5% of the genes in the input list belonging to this category. This is particularly interesting given that sorghum lines that have the stay-green trait are better able to survive under conditions of low water availability. The response to wounding category showed slight enrichment; however, there was no enrichment of genes associated with any of the other stimuli (Fig. 3).

The 42 genes that are involved specifically in the plant ‘response to osmotic stress’ were identified and their expression levels analysed (Fig. 4). This list contains genes encoding a dehydration-responsive element-binding (DREB1A) transcription factor, a ubiquitin ligase called salt and drought-induced RING finger 1 (SDIR1), and a CBL-interacting serine/threonine-protein kinase 1 (CIPK1). Other up-regulated genes include those encoding trehalose-6-phosphate synthase (TPS) and delta-1-pyrroline-5-carboxylate synthase (P5CS2), which are known to be important for the biosynthesis of trehalose and proline, respectively (Goddijn and van Dun, 1999; Ashraf and Foolad, 2007). Whilst not all of the enriched processes listed in Table 1 will necessarily be causal to the stay-green phenotype, it is possible that the higher expression of genes involved with the plant response to osmotic stress in B35 may be contributing to its ability to maintain green leaf area for longer under drought conditions.

Genes that are associated with the biosynthesis of proline are expressed at higher levels in the B35 stay-green line compared with a senescent variety

Amongst the 42 genes presented in Fig. 4, P5CS2 had the fifth highest fold change when comparing B35 and R16. The biosynthesis of proline is known to play an important role in the drought stress response (Ashraf and Foolad, 2007). Given the large difference in the expression of this gene (~8.7-fold), the proline biosynthesis pathway was investigated further. The SorghumCyc metabolic pathways database (http://pathway.gramene.org/gramene/sorghumcyc.shtml) was used to identify all sorghum genes involved in the biosynthesis of proline. The expression of these genes was then compared between B35 and R16 (Fig. 5). The expression of three genes associated with

![Fig. 1. Scatter plots showing the distribution of expression of filtered genes in the stay-green (B35) line compared with the senescent (R16) line. Axes denote normalized gene expression and squares represent individual genes. The green lines mark a 2-fold cut-off value. Differentially expressed genes are based on a 2-fold cut-off and a \(P\)-value <0.05. Colour corresponds to normalized gene expression, with red representing high relative expression and blue representing low relative gene expression.](http://jxb.oxfordjournals.org/Downloaded_from/at_UNIVERSITY_OF_DURHAM ON APRIL 17, 2016)
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**Fig. 2.** Comparison of fold changes obtained by microarray analysis and by qPCR. Bars represent the Log2 of the fold change when comparing expression in the stay-green (B35) and the senescent (R16) varieties.

**Table 1.** GO analysis of genes expressed at higher levels in B35 versus R16 (>2 fold; *P*-value<0.05)

| GO annotation | GO term                                      | Count in selection (out of 1038) | Count in total genome (out of 26 245) | Fold enrichment | *P*-value | FDR    |
|---------------|----------------------------------------------|----------------------------------|---------------------------------------|-----------------|-----------|--------|
| Biological process | Lipid localization                          | 7                                | 16                                     | 11.06           | 3.00E-07  | 0.00033 |
|                | Lead ion transport                           | 9                                | 33                                     | 6.90            | 6.70E-07  | 0.00055 |
|                | Post-embryonic morphogenesis                 | 16                               | 122                                    | 3.32            | 2.00E-06  | 0.0013  |
|                | Stamen morphogenesis                         | 9                                | 38                                     | 5.99            | 2.50E-06  | 0.0013  |
|                | Response to carbon dioxide                   | 7                                | 22                                     | 8.04            | 3.80E-06  | 0.0018  |
|                | Anatomical structure                         | 5                                | 10                                     | 12.64           | 7.30E-06  | 0.003   |
|                | Structural homeostasis                       | 5                                | 10                                     | 12.64           | 7.30E-06  | 0.003   |
|                | Response to endogenous stimulus             | 92                               | 1755                                   | 1.33            | 8.70E-06  | 0.0032  |
|                | Response to osmotic stress                  | 42                               | 631                                    | 1.68            | 1.20E-05  | 0.0038  |
|                | Androecium development                       | 15                               | 126                                    | 3.01            | 1.40E-05  | 0.0038  |
|                | Stamen development                           | 15                               | 126                                    | 3.01            | 1.40E-05  | 0.0038  |
|                | Floral organ formation                       | 11                               | 72                                     | 3.86            | 1.80E-05  | 0.0045  |
|                | Aminoglycan catabolic process                | 8                                | 39                                     | 5.19            | 2.80E-05  | 0.0061  |
|                | Chitin catabolic process                     | 8                                | 39                                     | 5.19            | 2.80E-05  | 0.0061  |
|                | Water transport                              | 5                                | 13                                     | 4.82            | 4.90E-05  | 0.0078  |
|                | Response to organic substance                | 104                              | 2117                                   | 1.24            | 3.40E-05  | 0.0067  |
|                | Chitin metabolic process                     | 8                                | 42                                     | 4.82            | 4.90E-05  | 0.0078  |
|                | Fluid transport                              | 5                                | 14                                     | 9.03            | 5.20E-05  | 0.0078  |
|                | Response to hormone stimulus                | 82                               | 1601                                   | 1.30            | 5.00E-05  | 0.0078  |
|                | Multidrug transport                          | 13                               | 109                                    | 3.02            | 4.90E-05  | 0.0078  |
|                | Drug transport                              | 14                               | 125                                    | 2.83            | 5.20E-05  | 0.0078  |
|                | Response to drug                             | 17                               | 185                                    | 2.32            | 0.00011   | 0.016   |
|                | Cellular amino acid derivative biosynthetic process | 31                          | 460                                    | 1.70            | 0.00012   | 0.016   |
|                | Response to chemical stimulus                | 145                              | 3244                                   | 1.13            | 0.00013   | 0.017   |
|                | Aminoglycan metabolic process                | 9                                | 61                                     | 3.73            | 0.00013   | 0.017   |
|                | Floral organ morphogenesis                   | 11                               | 90                                     | 3.09            | 0.00015   | 0.018   |
|                | Cell redox homeostasis                       | 15                               | 156                                    | 2.43            | 0.00016   | 0.019   |
|                | Response to biotic stimulus                  | 69                               | 1355                                   | 1.29            | 0.0002    | 0.021   |
|                | Corolla development                          | 9                                | 65                                     | 3.50            | 0.00022   | 0.021   |
|                | Flavonol metabolic process                   | 6                                | 28                                     | 5.42            | 0.00022   | 0.021   |

(Continued)
the biosynthesis of proline was found to be higher in B35 compared with R16 (Fig. 5). These genes correspond to two different P5CS2 transcripts (Sb03g039820.1 and Sb03g039820.2) and a glutamate S-semialdehyde dehydrogenase (Sb02g025790.1). These same genes were also expressed at higher levels in B35 at a younger seedling stage (Table 2).

The P5CS2 gene encodes an enzyme responsible for the rate-limiting step in proline biosynthesis (Kishor et al., 1995). Three P5CS genes have been identified in the sorghum genome. In
order to confirm that only one of these genes was expressed to higher levels in B35, qPCR, with probes designed specifically for each gene, was carried out and gene expression was compared in B35 and R16. Of the three annotated \( P5CS \) genes predicted in the sorghum genome, only \( P5CS2 \) (Sb03g039820.1) was expressed at higher levels in the B35 line (Fig. 6).
The B35 stay-green line has higher proline levels than the R16 senescent line under both well-watered and osmotically stressed conditions

Differences in gene expression do not always correlate with changes at the protein or metabolite level. Therefore, in order to determine whether the observed differences in P5CS2 gene expression result in an increase in actual proline levels, total proline content was quantified in B35 and R16 under both well-watered and osmotically stressed conditions using HPLC. Proline levels were found to be ~1.8-fold higher in the B35 stay-green line compared with the R16 senescent line under well-watered conditions and ~1.5-fold higher under osmotically stressed conditions (Fig. 7). The differences in P5CS gene expression therefore correlate well with the actual proline levels.

P5CS2 is found within a known QTL for the stay-green trait and contains an SNP in the promoter region

Genes within known QTLs for the stay-green trait were identified using the CSGR (http://helos.pgml.uga.edu/qtl/). The majority of the stay-green QTLs in this database were identified using B35 as the source of stay-green (Tuinstra et al., 1997; Crasta et al., 1999; Subudhi et al., 2000; Xu et al., 2000b; Sanchez et al., 2002; Harris et al., 2007). This list of genes was compared with the list of differentially expressed genes identified in the microarrays. Out of the 2036 differentially expressed genes identified in the arrays, 289 are within a known QTL for stay-green (see Supplementary Tables S5 and S6 at JXB online for a full list). Interestingly for this study, P5CS2 also lies within a stay-green QTL region that is now known as Stg1 (Subudhi et al., 2000; Xu et al., 2000b).

Differences in the expression of P5CS2 between the varieties could be associated with polymorphisms in the upstream promoter region. To test this, 500 bp upstream of the start codon was amplified using PCR and then sequenced. The sequences from the stay-green B35 variety and two senescent varieties, R16 and Tx7000, were then compared (Fig. 8). Sequence alignment identified three single nucleotide polymorphisms (SNPs) and a 22 bp deletion within the B35 line when compared with both senescent varieties (Fig. 8). Two of the identified SNPs lie within known cis-element motifs. For example, a G to C SNP can be found in a potential C-box motif (Simpson et al., 2003) and an A to C SNP in a YACT motif (Gowik et al., 2004). A potential Myb element within the B35 upstream sequence is not present in the senescent R16 and Tx7000 varieties (Grotewold et al., 1994). It is therefore possible that differences in the promoter sequence of P5CS2 in B35 may be responsible for the differences in the expression of this gene.

Discussion

The identification of genes conferring drought tolerance and in particular those underlying specific traits for drought tolerance will be very important in the future in order to reduce the adverse impacts of drought stress on crop yields. In previous studies, transcriptomic analysis has proven to be a powerful tool for the identification of stress-induced genes in sorghum (Buchanan et al., 2005; Salzman et al., 2005; Dugas et al., 2011; Johnson et al., 2014). Here, microarray analysis was used to compare gene expression between a drought-tolerant stay-green sorghum line and a drought-sensitive senescent line in order to identify genes and biological processes that are putatively associated with the important stay-green trait. Gene expression was compared under well-watered conditions because many previously observed differences in physiology between stay-green and senescent lines were identified under non-stressed conditions (i.e. differences in chlorophyll content and transpiration efficiency) (Kassahun et al., 2010; Vadez et al., 2011; Borrell et al., 2014).

A total of 1038 genes were found that are expressed at higher levels in the stay-green line and 998 genes that are

Table 2. Genes associated with proline biosynthesis that are expressed at higher levels in B35 versus R16

| SBID          | Gene name                                      | FC (Abs) in B35 versus R16 at 50 DAS | FC (Abs) in B35 versus R16 at 14 DAS |
|---------------|------------------------------------------------|-------------------------------------|-------------------------------------|
| Sb03g039820.1 | Delta1-pyrroline-5-carboxylase-synthetase (P5CS2) | 8.74                                | 2.85                                |
| Sb03g039820.2 | Delta1-pyrroline-5-carboxylase-synthetase (P5CS2) | 8.55                                | 2.52                                |
| Sb02g025790.1 | Glutamate S-semialdehyde dehydrogenase          | 2.32                                | 3.35                                |
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Expressed at lower levels (Fig. 1). Whilst not all of these will necessarily be causal to the stay-green phenotype, an analysis of these genes and the processes they are involved with provides a valuable insight into the mechanisms underlying the trait. Ontological analysis of these genes identified enriched biological processes, including ‘response to osmotic stress’ and ‘water transport’ (Table 1). Stay-green sorghum lines are known to be able to survive for longer under conditions of low water availability (Rosenow et al., 1983; Xu et al., 2000a). For this reason, genes that are known to provide protection against these conditions, such as those in the ‘response to osmotic stress’ category, provide obvious initial candidates as contributors to the phenotype.

An interesting gene found within the ‘response to osmotic stress’ GO category was P5CS2. The products of P5CS genes are known to be involved in the conversion of glutamate to pyroline-5-carboxylate and as such they act as the rate-limiting step in the biosynthesis of proline (Kishor et al., 1995). Not only was the expression of this gene shown to be higher in the stay-green line but this higher expression resulted in constitutively higher levels of actual proline levels (Fig. 7).

Proline is known to accumulate in plants, including sorghum, under low water conditions, and is known to have a number of protective properties including a role in osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins (Wood et al., 1996; Hsu et al., 2003; Ashraf and Foolad, 2007; Su et al., 2011; Damame et al., 2014). In addition, evidence has suggested that proline is able to induce the expression of stress-responsive genes which possess proline-responsive elements (PREs) in their promoters (Satoh et al., 2002). As a consequence, the overexpression of P5CS genes and the accumulation of proline have been shown to result in drought tolerance in a wide range of species (Kishor et al., 1995; Hong et al., 2000). In addition, proline concentrations have also been shown to be generally higher in stress-tolerant genotypes of plants, including sorghum (Sivaramakrishnan, 1988; Hsu et al., 2003; Nayyar and Walia, 2003; Ashraf and Foolad, 2007; Damame et al., 2014). The proline accumulation identified here could therefore be an important way in which stay-green lines are able to withstand drought stress for longer and therefore maintain their green leaf area.

The maintenance of cell turgor via osmotic adjustment is particularly important during cell growth and leaf expansion. Sorghum plants with a better capacity for osmotic adjustment have been shown to have a larger leaf area and have better leaf retention during grain filling (Tangpremsri et al., 1995). Stay-green sorghum lines have been shown to have higher RWC than senescent lines (Xu et al., 2000a). It is possible, therefore, that the high proline accumulation identified here in the stay-green variety is contributing to the maintenance of high RWC. This could help with the production of a strong canopy that is better able to intercept light. This means that when fresh water availability is reduced at later growth stages, the stay-green varieties are better adapted to cope. Some evidence has suggested that stay-green lines have a modified root architecture (Borrell et al., 2014a). Whilst not investigated specifically in this study, it is also plausible
Numerous studies have led to the identification of QTLs for the stay-green trait, and four of these QTLs are known to be consistent across multiple environments (Crasta et al., 1999; Xu et al., 2000a; Sanchez et al., 2002). Here, the genes that were differentially expressed between the stay-green and senescent varieties were compared with genes known to be within the Stg QTL regions in the genome. This approach of combining QTL and microarray analysis is a powerful one for the identification of candidate genes for a trait and has contributed significantly to candidate gene identification (Pandit et al., 2010; Yano et al., 2012). Out of the 2036 differentially expressed genes, 286 were found to lie within a stay-green QTL region. Whilst not all of the differentially expressed genes are within the QTL intervals, and so clearly not candidates, many may act downstream of these genes and therefore could act as diagnostic markers for trait selection. Interestingly, the P5CS2 gene previously described can be found within the Stg1 QTL interval. Sequencing of the putative P5CS2 promoter in both stay-green and senescent varieties enabled the identification of three SNPs within the B35 promoter along with a 22 bp deletion (Fig. 8). These

that if P5CS2 is additionally expressed at higher levels in the roots, osmotic adjustment could enable better root growth, which could facilitate water uptake. Stay-green sorghum plants are able to save water in the period prior to flowering (Xu et al., 2000a). The accumulation of compatible solutes such as proline could again be associated with conferring these phenotypes.

Given the complexity of the stay-green trait, however, it is perhaps unlikely that only one gene or process is involved in conferring the trait. Indeed, at least four QTLs have been identified, and individual introgressions of these QTLs all show the stay-green phenotype, albeit to different extents (Subudhi et al., 2000; Sanchez et al., 2002; Kassahun et al., 2010; Vadez et al., 2011). There are therefore at least four different contributing genetic regions. Studies by Borrell et al. suggest that developmental differences could be a contributing factor. For example, stay-green near-isogenic lines (NILs) have been shown to display reduced tillering and reduced size of upper leaves when compared with their recurrent parent Tx7000 (Borrell et al., 2014a). These differences are thought to reduce crop water usage prior to anthesis, resulting in greater water availability at the post-flowering stage. Interestingly, the present results show an enrichment of the ‘anatomical structure homeostasis’ and ‘post-embryonic morphogenesis’ GO categories amongst the highly expressed genes. Within these categories are genes associated with auxin biosynthesis and transport (see Supplementary Table S2 at JXB online). The plant hormone auxin is known to play a role in shoot branching and leaf development (Dengler and Kang, 2001; McSteen and Leyser, 2005); therefore, it is possible that these identified auxin-related gene expression differences may be contributing to the developmental differences previously described.

Multiple genes may be acting co-operatively to bring about the stay-green phenotype. Other interesting genes identified in this study include the signalling gene SDIR1. Homologues of this SDIR1 gene have been shown in Arabidopsis, maize, and rice to confer drought tolerance when overexpressed by influencing stomatal aperture and water loss by transpiration (Zhang et al., 2007, 2008; Xia et al., 2012). Given the known differences in transpiration efficiency and pre-anthesis water usage between stay-green and senescent lines (Xu et al., 2000a; Vadez et al., 2011), it is possible that higher levels of SDIR1 are resulting in reduced transpiration and could therefore be contributing to this aspect of the stay-green phenotype. The DREB1A transcription factor gene was also expressed at higher levels in the stay-green line. Transcription factors act as master regulators and can influence the expression of numerous downstream genes. DREB transcription factors have been shown in a number of species to influence stress-related gene expression, and their overexpression has been shown to increase stress tolerance in wheat and rice (Ito et al., 2003; Shen et al., 2003).

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polymorphisms co-locate with known cis-element motifs. For example, a G to C SNP was identified within an ABRE-like sequence, ACGTG, which is known to be involved in the induction of genes associated with the response to dehydration stress (Simpson et al., 2003). The A to C SNP also lies within a YACT motif (Gowik et al., 2004). It is possible that these polymorphisms underlie the QTL and are responsible for the differences in the expression of P5CS2 between the stay-green and senescent varieties.

Further verification of candidate genes in the other QTL regions, by genome sequencing and by the analysis of gene function though the production of transgenics, will be an important next step to validate their importance. It is important to bear in mind here that the gene expression comparison described is between only two genotypes; one stay-green and one senescent. B35 was selected as a source of stay-green for this study due to the fact that the majority of QTL mapping experiments have used this line (Tuinstra et al., 1997; Subudhi et al., 2000; Xu et al., 2000b). However, it will be important to investigate gene expression changes in other stay-green sources. In particular, the use of NILs which differ genetically in one or more QTL only will be particularly beneficial for the validation of candidate genes. Some evidence suggests that some Stg QTLs in sorghum overlap with QTLs for nodal root angle, and it has been suggested that this could enhance water uptake in the stay-green lines (Mace et al., 2012; Borrell et al., 2014a). It will also therefore be important to investigate the role of the roots and root signalling in conferring the phenotype. The stay-green trait in sorghum is an extremely complex phenotype which is confounded by strong genotype and environment interactions (Vadez et al., 2011); therefore, it will also be important to investigate gene expression changes at different developmental stages, in different growth environments, and in different genetic backgrounds.

In conclusion, these results provide an excellent starting point for the identification of genes underlying this important agricultural trait and could help facilitate breeding for stay-green via MAS or via the production of more targeting introgression lines in the future.
Supplementary data

Supplementary data are available at JXB online Figure S1. Measurements of $F_{v}/F_{m}$ in R16 and B35 from 35 DAS. Samples were taken for the microarray analysis at ~45 DAS.

Table S1. qPCR probes used for validation of the microarray data.

Table S2. Genes expressed to higher levels (FC>2, $P<0.05$) in B35 versus R16 at 50 DAS.

Table S3. Genes expressed to lower levels (FC>2, $P<0.05$) in B35 versus R16 at 50 DAS.

Table S4. B35 versus R16 gene expression changes obtained using qPCR and microarray analysis.

Table S5. Genes that are expressed to higher levels (FC>2, $P<0.05$) in B35 versus R16 at 50 DAS and that lie within the Stg QTL region.

Table S6. Genes that are expressed to lower levels (FC>2, $P<0.05$) in B35 versus R16 at 50 DAS and that lie within the Stg QTL region.

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