LARGE-SCALE BIOLOGY ARTICLE

Time-series transcriptomics reveals that *AGAMOUS-LIKE22* affects primary metabolism and developmental processes in drought-stressed Arabidopsis

Running title: Temporal drought dynamics in Arabidopsis

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Synopsis: Temporal transcriptome analysis during drought stress coupled with Bayesian network modelling reveals early drought signalling events, and identifies AGL22 as a regulator of primary metabolism.

Abstract

In *Arabidopsis thaliana*, changes in metabolism and gene expression drive increased drought tolerance and initiate diverse drought avoidance and escape responses. To address regulatory processes that link these responses, we set out to identify genes that govern early responses to drought. To do this, a high-resolution time series transcriptomics dataset was produced, coupled with detailed physiological and metabolic analyses of plants subjected to a slow transition from well-watered to drought conditions. A total of 1815 drought-responsive differentially expressed genes were identified. The early changes in gene expression coincided with a drop in carbon assimilation, and only in the late stages with an increase in foliar abscisic acid content. To identify gene regulatory networks (GRNs) mediating the transition between the early and late stages of drought, we used Bayesian network modelling of differentially expressed transcription factor (TF) genes. This approach identified *AGAMOUS-LIKE*22 as key hub gene in a TF GRN. It has previously been shown that *AGL22* is involved in the transition from vegetative state to flowering but here we show that *AGL22* expression influences steady state photosynthetic rates and lifetime water use. This suggests that *AGL22* uniquely regulates a transcriptional network during drought stress, linking changes in primary metabolism and the initiation of stress responses.

INTRODUCTION

Water limitation in agriculture is poised to intensify in the coming decades due to urbanisation, industrialisation, depletion of aquifers and increasingly erratic rainfall patterns exacerbated by climate change (Easterling et al., 2000; Christensen et al., 2007; Seager et al., 2007; Famiglietti and Rodell, 2013). Reduced water availability leads to drought stress, which is a major constraint on the physiology, growth, development and productivity of plants (Boyer, 1970; 1982; Verelst et al., 2013; Skirycz et al., 2010; Lobell et al., 2011; Lobell and Field, 2007; Schlenker and Roberts, 2009). Therefore, understanding the mechanisms of drought response in plants is essential for the improvement of plant performance under water-limiting conditions and has been the subject of many investigations over the years (Nakashima et al., 2009; Shinozaki and Yamaguchi-Shinozaki, 1997; 2007; Chaves et al., 2009; Pinheiro and Chaves, 2011). Water deficit responses are complex and require stress sensing and signalling to adjust plant growth, maintain water status through osmoregulation, prevent water loss through decreases in stomatal conductance and activate detoxification processes (Passioura, 1996; Chaves et al., 2003; Pinheiro and Chaves, 2011). An important consideration is that even a slight reduction in water availability can elicit stomatal closure and a reduction in CO_2 assimilation, and in combination with the diversion of resources towards drought defence mechanisms will affect plant productivity (Chaves et al., 2003).

Plants have adopted different strategies to respond to water limitation, such as drought escape through early flowering and reducing the size of plants to increase water use efficiency, or drought avoidance through enhanced soil moisture capture or reduced transpiration (Ludlow, 1989; Blum, 2005; Aguirrezabal et al., 2006; Franks, 2011). In this context, the influence of drought on plant development and growth through its effects on developmental processes such as germination, seedling growth and leaf development has been studied extensively in the past decade (Finkelstein et al., 2002; van der Weele et al., 2000; Xiong et al., 2006; Yaish et al., 2011). Optimal timing of flowering and inflorescence development are important traits

110 essential in determining plant yield, and these can vary greatly in response to
111 water limitation (Ma et al., 2014, Su et al., 2013; Eckhart et al., 2004; Franke
112 et al., 2006).
113 At the cellular level, plants respond to drought with changes in gene
114 expression, protein and metabolite abundances (Baerenfaller et al., 2012;
115 Wilkins et al., 2010, Harb et al., 2010; Charlton et al., 2008), which are part of
116 defence mechanisms and detoxification processes (Begcy et al., 2011;
117 Ozfidan et al., 2011; Shinozaki and Yamaguchi-Shinozaki,. 2007). Recent
118 progress in genomics, transcriptomics and bioinformatics has paved the way
119 for dissecting drought-response mechanisms and has enabled the targeted
120 manipulation of drought-responsive genes in plants. For example, the
121 overexpression of a number of genes that code for transcription factors (TFs)
122 leads to drought resistance (Tang et al., 2012; Quan et al., 2010; Chen et al.,
123 2008; Nelson et al., 2007; Sakuma et al., 2006).
124 In many studies to identify genes important in the regulation of drought
125 responses, the effects of water limitation at the transcriptional level have been
126 analysed by exposing plants to severe dehydration. This involves treatments
127 such as cutting and air drying leaves and/or roots, or induction of osmotic
128 shock through the application of highly concentrated osmotica such as
129 polyethylene glycol (PEG) or mannitol (Seki et al., 2002; Kreps et al., 2002;
130 Kawaguchi et al., 2004; Kilian et al., 2007; Weston et al., 2008; Fujita et al.,
131 2009; Mizogushi et al., 2010; Deyholos, 2010; Abdeen et al., 2010). These
132 experiments have substantially increased our knowledge of molecular
133 responses under severe drought stress, but they do not always reflect
134 physiological conditions experienced by drought-stressed soil-grown plants
135 (Wilkins et al., 2010; Harb et al., 2010; Bechtold et al., 2010; 2013; Lawlor,
136 2013; Zhang et al., 2014). Physiological responses such as stomatal
137 conductance, photosynthetic performance and metabolic changes are usually
138 not measured during the progression of the drought stress, and the varied
139 nature of the stress induction treatments makes comparative analysis
140 between experiments problematic. Slow developing soil water deficits have
141 different physiological consequences than those induced by rapid tissue

142 dehydration and therefore possibly utilize different gene networks (Chaves et
143 al., 2003; 2009; Pinheiro and Chaves, 2011).
144 This inconsistency amongst experiments was first noted in a meta-analysis of
145 microarray experiments comparing air drying, soil drying and mannitol
146 treatments (Bray et al., 2004). This analysis found very few differentially
147 expressed genes (DEGs) common to all treatments (Bray et al., 2004).
148 Consequently, recent experiments have focussed on soil-grown plants
149 (Wilkins et al., 2010; Harb et al., 2010; Zhang et al., 2014). From these
150 studies, an overall integrative picture of the temporal responses to drought is
151 emerging slowly, and it is clear that use of a single or a small number of time
152 points and different types of experimental conditions lead to very different
153 outcomes. One consequence of this is that very little is known about the early
154 events in the perception of drought stress signals (Ueguchi et al., 2001;
155 Wohlbach et al., 2008; Pinheiro and Chaves 2011).
156 To address the above issues, we set out to gain detailed information on the
157 processes that occur during the transition from well-watered to drought
158 conditions, in which the intensity of the stress becomes gradually greater. We
159 monitored the physiological and metabolic status of plants through a
160 progressive drought experiment and mapped onto these data the temporal
161 responses of the transcriptome. Our intention was to use the highly resolved
162 transcriptional profiling data to construct gene regulatory networks (GRNs)
163 using dynamic Bayesian network modelling (Beal et al., 2005; Breeze et al.,
164 2011; Penfold and Wild, 2011) with the aim of identifying regulatory genes
165 functional during drought perception and signalling. The goal was to link early
166 physiological and metabolic drought avoidance responses with later drought
167 escape and/or tolerance responses (Claeys and Inze, 2013). This initially
168 required testing of the network modelling to evaluate the capability of these
169 approaches to identify genes important in the regulation of drought responses.
170 This was achieved by selecting a highly connected candidate gene,
171 *AGAMOUS-LIKE22* (*AGL22*) from the GRNs. *AGL22* has an established
172 function in plant development (Mendez-Vigo et al., 2013, Gregis et al 2013),
173 but in this study it was shown to play a thus far undiscovered role in the

critical early stages of the plant's response to drought. These results demonstrated the potential value of experimental strategies that combine time series transcriptomics data with dynamic modelling as a means of identifying stress-responsive genes.

RESULTS

Time-series experiments were carried out analyzing physiological, metabolic and transcriptional changes in Arabidopsis to reveal the chronology of plant responses to drought stress. A progressive slow-drying experiment starting at 95% relative gravimetric soil water content (rSWC) and drying down to 17% rSWC was carried out on 5-week-old Arabidopsis plants (Figure 1). To determine the severity of the stress, daily measurements of relative leaf water (RWC) content (Figure 1A) and leaf water potential were also carried out (Figure 1B). During the experiment, the average rSWC loss was approximately 10% per day, but RWC was maintained throughout the progressive drying period until the point of wilting at 17% rSWC (Figure 1A).

Maximum photosynthetic capacity responds similarly in well-watered and drought-stressed plants during a progressive drought experiment

Stomatal conductance (g_s) and photosynthetic carbon assimilation (A) were measured daily on well-watered and drought-stressed plants through the progressive drought treatment (Figure 1C and Figure 1D). Stomatal conductance declined at ~60% rSWC (day 5; Figure 1C), which was followed by a decline in carbon assimilation at ~45% rSWC (day 7), indicating that stomatal diffusional limitations affected carbon assimilation (Figure 1D). Plant growth evaluated as rosette fresh weight and rosette area ceased at approximately 40% rSWC (Supplemental Figure 1A-C).

The light and CO_2-saturated maximum photosynthetic rate (A_{max}), maximum rate of carboxylation (V_{Cmax}) and the rate of Ribulose-1,5-bisphosphate (RuBP) regeneration (J_{max}) showed no difference between well-watered and drought-stressed plants (Figure 2A). In addition, maximum- and operating-efficiencies of photosystem II (Fv/Fm, Fv'/Fm' and Fq'/Fm'; Baker, 2008)

showed no change during the drought period (Figure 2B and Supplemental Figure 1D), suggesting that the overall primary metabolic capacity was maintained as drought conditions progressed. The sequential changes in photosynthetic physiology, relative water content and leaf water potential suggest that these conditions allowed us to capture the transition between early physiological changes and later stress responses.

Metabolite profiling indicates the stable nature of primary metabolism during drought stress

The decline in stomatal conductance and carbon assimilation led us to perform metabolite analysis to evaluate changes in primary and secondary metabolism (Figure 3). Untargeted LC-MS metabolite profiling was carried out on samples harvested at early (day 2, ~80% rSWC), mid (day 7, ~50% rSWC) and late (day 13, 17% rSWC) stages of the drought stress. This analysis showed that the majority of the metabolome was unchanged throughout most of the drought treatment, and only by the final day of drought stress (17% rSWC) distinct clustering between well-watered and drought-stressed samples emerged (Figure 3A). Leaf development was a major factor for sample separation, with days clustered more closely together than treatments (Figure 3A).

Targeted metabolite analysis was carried out to determine the foliar levels of 102 stress-associated compounds (Supplemental Data Set 1, Supplemental Data Set 2 and Supplemental Data Set 3), which also revealed a mainly late response for many of these stress-associated metabolites (Supplemental Data Set 1, Supplemental Data Set 2 and Supplemental Data Set 3). Often these changes were limited to the last 2-3 time points (between ~30% - 17% rSWC; Supplemental Figure 2 and Supplemental Figure 3). For example, metabolites indicative of drought stress increased only during the late stages of the dehydration period (Figure 3B and Figure 3C). There was a significant increase in ABA levels during the last 4 time points (Xiong et al 2002; Figure 3B), while proline, a drought stress-responsive compatible solute in vascular plants (Sperdouli and Moustakas, 2012), accumulated to significant levels

only during the last two time points (Figure 3B). Additionally, the accumulation of secondary metabolites commonly associated with stress responses such as anthocyanins and flavonols were altered during the late stages of the drought response (Sperdouli and Moustakas, 2012; Supplemental Data Sets 1 and 3; Supplemental Figure 3). Oligosaccharides/disaccharides associated with osmotic protection during drought and osmotic stresses (Galactinol and Raffinose; Taji et al., 2002) significantly accumulated during the last 4 days of the drought response (Figure 3C, Supplemental Data Set 1). In conclusion, leaf metabolism remained largely stable during the first 9 days of the experiment, while changes previously associated with drought stress (Xiong et al 2002; Taji et al 2002; Sperdouli and Moustakas, 2012), only became evident during the last 3-4 time points.

Transcriptomics analysis on a single leaf identifies 1815 differentially expressed genes (DEGs) during progressive drought stress

Transcriptome profiling was carried out on leaf 7 to integrate the complex physiological and metabolic responses with changes at the gene expression level. Leaf 7 was fully expanded at the time of the experiment (Supplemental Figure 1C), and was chosen because a detailed temporal transcriptome analysis of leaf development was available (Breeze et al., 2011). Single leaf 7 samples for transcriptome analysis were taken each day at the midpoint of the light period. RNA from four leaf samples per treatment and time point was hybridized on CATMA v4 arrays (Sclep et al., 2007, see Methods). An adapted MAANOVA (MicroArray ANalysis Of VAriance) method was used to analyze the data for each comparison (Wu et al., 2003; Churchill, 2004; Breeze et al., 2011; Windram et al., 2012). This generated a single normalized expression value for each gene. A Gaussian process two-sample test (GP2S; Stegle et al., 2010) was used to identify DEGs. Choosing a Bayes Factor (BF) value (likelihood of differential expression) of >6, resulted in a total of 1815 DEGs (Supplemental Data Set 4). The up-regulated group of genes showed on over-representation of GO terms related to carbohydrate biosynthesis, flavonoid and secondary metabolic processes, while down-

regulated genes were enriched in protein translation, cell wall-associated processes, pigment biosynthesis and chloroplast associated processes (Supplemental Data Set 5).

GO terms related to stress, dehydration and hormonal regulation, including ABA were not enriched in the complete dataset. This result suggested that the overall progressive drought experiment was not a severe dehydration stress response, as indicated by the maintenance of primary metabolic capacity (Figure 2) as well as the late responses of stress-associated metabolites (Figure 3). Leaf water potential has been used as a measure of the progression and effect of drought stress on plants (Zhang et al 2014). We estimated the cumulative number of DEGs at each time point by determining the time of first differential expression (TOFDE) for each gene (Supplemental Data Set 6, Figure 4A). In our experiment, leaf water potential correlated significantly with rSWC (Figure 4B) as well as the number of DEGs (Figure 4C) and showed a weaker correlation with carbon assimilation (Figure 4D). The biggest drop in leaf water potential occurred between 40% (day 8) and ~30% (day 9) rSWC, which coincided with the biggest increase in DEGs (Figure 4A and Figure 4C), potentially indicating a shift from mild to severe drought stress.

To evaluate the transcriptome dataset in the context of other drought experiments, we also compared our dataset to two soil-based drought studies in Arabidopsis. The first experiment carried out by Harb et al (2010) comprised a microarray comparison of leaf samples under moderate drought stress (maintaining soil water content at 30% of field capacity) and progressive drought stress at the pre-wilting (~15% field capacity) and wilting (~10% field capacity) stages. In the progressive drought stress treatment, 3005 genes responded >2- and <0.5 fold, in comparison to 441 genes for the moderate drought treatments (Supplemental Data Set 7). The second study analysed samples at a loss of 25% soil water of field capacity measured at 6-hour intervals across a 24-hour period (Wilkins et al., 2010), and identified 570 genes that responded across a 24-hour interval (hereafter called diel; Supplemental Data Set 7). A general overview of overlapping genes between

the different experiments showed that only 30 genes were common to all 4 treatments (Figure 5A). Among the overlapping genes were known stress-responsive, ABA-responsive and secondary metabolism genes, which predominantly responded during the latter half of the drought experiment (Supplemental Data Set 8). While there were common elements in all four treatments, 63% of the DEGs in our dataset were unique to the time-series (Figure 5A) and were potentially related to the adjustments to early and moderate drought stress.

Slow soil drying induces a senescence response in leaf 7

To assess developmental changes in leaf 7 during drought stress we compared the drought time series to an Arabidopsis leaf 7 senescence time-series dataset (Breeze et al., 2011; Supplemental Data Set 7). In all, 842 genes overlapped with the senescence dataset (p-hyper: 4.4E-135; Figure 5B), of which 83% responded between days 8 and 13 (40% - 17% rSWC; Supplemental Data Set 9). The overlap contained genes associated with oxidation/reduction-related processes, pigment biosynthesis and primary metabolism, which were predominantly down-regulated during drought stress (Figure 5C and Figure 5D; Supplemental Data Set 10). The induction of senescence-related processes during drought stress is a known phenomenon (Munné-Bosch and Alegre, 2004), and was further confirmed by a significant overlap of genes between the published drought and senescence datasets (Supplemental Data Set 7; Supplemental Figure 4A).

In addition, changes in the expression of secondary metabolism genes were observed in the drought time-series (Figure 5E), including *CHALCONE SYNTHASE* (*CHS*), *FLAVONOL SYNTHASE1* (*FLS1*), *LEUCOANTHOCYANIDIN DIOXYGENASE* (*LDOX1*), *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), and *ANTHOCYANINLESS 2* (*ANL2*; Supplemental Figure 5; Supplemental Data Set 9). This coincided with increased accumulation of flavonol and anthocyanin (from day 11; Supplemental Data Set 1, Supplemental Figure 3), and suggested that the plants had entered a severe stress phase (Vanderauwera et al., 2005).

Therefore, it was concluded that slow soil drying induces senescence in leaf 7 but only at the point of severe drought stress. By contrast, early responses to soil drying (days 1-7) were mostly unique to the drought time series. Importantly for later considerations, the induction of leaf senescence in response to drought did not affect flowering time (Supplemental Figure 4B).

Temporal clustering reveals co-regulated groups of genes, but does not reveal specific regulatory mechanisms

The cumulative number of DEGs at each time point (Supplemental Data Set 6) confirmed that major gene expression changes occurred late during the drought experiment as the number of genes that showed first differential expression at each time point was highest between days 8 - 11 (Figure 4A). 336 genes responded during the first half of the experiment, while the majority of genes (1479) showed first differential expression during the latter half of the experiment. A Euclidean distance matrix of the average expression values of the four biological replicates for each dataset was generated and used in hierarchical cluster analysis. The resulting dendrogram showed that samples clustered in relation to treatments and within the drought treatment into early and late stage responses (Figure 6A). Dividing the dataset into early (days 1 to 7, ~95% - 45 % rSWC) and late responses (days 8 to 13, ~40% - 17% rSWC), also revealed functional groups of genes responding at different times throughout the progressive drought. Early up-regulated genes were associated with carbohydrate and glycoside biosynthetic processes, general carbohydrate metabolic processes and inorganic cation transporter activities (Table 1). The late up-regulated genes encompassed flavonoid and secondary metabolite biosynthesis, while the late down-regulated genes were involved in translation, pigment biosynthesis, photosynthesis-related processes and oxidation/reduction processes (Table 1). To gain insight into the molecular factors underlying this temporal separation of samples, hierarchical cluster analysis of the DEGs was carried out using SplineCluster (Heard et al., 2005) on the basis of gene expression patterns in the drought stressed leaf only. Using a prior precision value of 0.01, the 1815 genes were

366 divided into 28 clusters (Figures 6B and Figure 7, Supplemental Data Set 11).
367 The first 14 clusters showed an overall up-regulation and contained 1149
368 genes, while the last 14 down-regulated clusters contained 667 genes (Figure
369 7, Supplemental Data Set 11). The 28 clusters were hypothesised to
370 represent groups of genes that are co-regulated during the drought
371 experiment. To explore potential regulatory mechanisms of genes clustered in
372 specific temporal expression profiles we analyzed each individual cluster for
373 over- and under-representation of GO terms in the Biological Process and
374 Molecular Function, and for over-representation of known TF binding motifs in
375 promoters (Supplemental Data Set 12 and Supplemental Data Set 13;
376 Supplemental Figure 6). A few clusters showed enrichment of expected GO
377 terms in response to drought, such as flavonoid biosynthesis, photosynthesis,
378 pigment biosynthesis and response to stress (Supplemental Data Set 12,
379 Figure 7). Most clusters however did not show any enrichment or under-
380 representation of GO terms (Supplemental Data Set 12, Figure 7), and only 2
381 clusters (cluster 1 and 9) contained the ABRE binding motif, known to
382 perceive ABA-mediated drought and osmotic stress signals (Supplemental
383 Figure 6, Supplemental Data Set 13; Kim et al., 2011).
384
385 **Bayesian state space modelling identifies genes that link drought**
386 **responses to plant development through regulating transcriptional**
387 **networks**
388 The data presented so far did not allow us to draw conclusions about specific
389 regulatory mechanism across the whole time series, but suggested that early
390 and late responses to a decline in soil water content are regulated differently.
391 Large-scale transcriptional re-programming and metabolic adjustment did not
392 play a dominant role during the early phases of the dehydration response.
393 Nevertheless, among the 337 DEGs responding between 95% and 40%
394 rSWC were 33 TF genes (Supplemental Data Set 14), of which 25% had a
395 functional annotation of development (GO:0032502; Supplemental Data Set
396 14). This suggested that a re-programming of developmental processes
397 during drought stress may have occurred in response to the observed early

physiological changes (closure of stomata, reduction in leaf water potential). We reasoned that the expression of early TF genes must therefore play a role in orchestrating this acclimation to drought stress.

Metropolis Variational Bayesian State Space Modelling (M-VBSSM; Penfold, University of Warwick, see Supplemental Methods) was initially performed on 176 differentially expressed TFs in the dataset (Supplemental Data Set 14). This approach selects subsets of TFs to generate a network, which is continually updated by probabilistic replacement of TFs to generate a series of networks and provides a consensus model based on the marginal likelihood (see Supplemental Methods). From the consensus model, we could calculate the occurrence of each TF (the number of times a particular TF appeared over all models), and a count of the number of downstream connections each TF had across all models at a particular z-score, in this case indicating a 95% confidence threshold (Supplemental Data Set 15). TFs that scored well in both rankings were deemed highly connected hubs with many significant edges. The M-VBSSM consensus model indicated that developmental genes played an important role in the regulation of drought, as 4 out of the top 10 highly connected TFs were associated with the regulation of plant development (Supplemental Data Set 15). In addition, two of the top 10 TFs were amongst the group of early responding TFs (Supplemental Data Set 14; Supplemental Data Set 15). Whilst the consensus model was useful for the initial ranking of genes, it did not represent a causal model, instead representing a type of averaging of many different network models. For this reason we opted to model a smaller selection of genes, which was advantageous for two reasons. Firstly, the final model was smaller and sparser, and therefore more interpretable, and secondly, the resultant interactions could be interpreted causally.

Therefore the top 10 “hub” TFs with the highest frequency of occurrence and 90 random transcription factors were chosen for analysis with the VBSSM package (Beal et al., 2005; Supplemental Data Set 16). As part of this selection, we also chose early and late responding TFs. In total, 19 early responding TFs (days 1 – 7) were included in the model, to establish a

potential transcriptional link between early and late responses (Supplemental Data Set 16).

The resultant model placed the early-responding transcription-factor gene *AGL22* at the centre of a 25 TF gene network (Figure 8A). *AGL22* was differentially expressed in the drought experiment beginning at day 5 (Supplemental Figure 7A). Fifteen TF genes that were part of the GRN were initially analysed by qPCR to check for differential expression under drought in wild-type plants (20% rSWC). All genes were differentially expressed in line with the levels observed in the microarray experiment, except for *PACLOBUTRAZOL RESISTANCE1* (*PRE1*), *BASIC HELIX-LOOP-HELIX038* (*BHLH038*) and *AUXIN RESPONSE FACTOR1* (*ARF1*; Figure 8B, Supplemental Figure 7B).

AGL22 is known to affect flowering time and plant development (Supplemental Figure 7C; Mendez-Vigo et al., 2013, Gregis et al., 2013); however, it was not regulated during leaf senescence (Supplemental Data Set 9), suggesting that *AGL22* uniquely regulated a transcriptional network during drought stress. This was further explored by performing VBSSM using the control time-series data set of the same transcription factor genes. If *AGL22* was a hub gene in the control time series, it would suggest a role in developmental reprogramming over the 13 day experimental period regardless of drought stress. The Bayesian modelling resulted in a number of fragmented connections of a small number of genes (Supplemental Figure 8A), suggesting these genes were not part of a gene regulatory network under well-watered conditions. The highly connected genes in the drought model, *AGL22* and *RAP2.12* did not feature, not even as peripheral genes (Supplemental Figure 8A).

Therefore, the early responding TF *AGL22* was chosen for further analysis to establish how far an unbiased modelling approach can be used to identify genes capable of influencing plant drought phenotypes and downstream network connections. Two independent T-DNA insertion lines were isolated (see Methods), both of which were confirmed knockout mutants for *AGL22*

(Figure 8C, Supplemental Figure 8B). The mutants were subsequently analysed for their effect on the *AGL22*-centered network interactions after drought stress. Eight out of fifteen TF genes differentially expressed under drought conditions exhibited altered gene expression in at least one of the *agl22* mutants compared to the wild type (Figure 8D). This implied that ~50% of the network connections were regulated at least partially through *AGL22*, but also suggested the possibility of redundancy within the network (Figure 8A, D). Four late TFs (*WRKY20*, *GIS*, *DREB1A* and *FBH3*) were substantially down-regulated in both agl22 mutants after drought, suggesting that these TFs were primarily regulated through *AGL22* (Figure 8D).

Both *agl22* mutants had early-flowering, fast-drying drought escape phenotypes

It is important to note that due to the early-flowering phenotype of the *agl22* mutants (Supplemental Figure 7C), drought stress was begun at day 22 after sowing, when there was no visible differences in rosette leaf number (Supplemental Figure 9A, Supplemental Figure 9B and Supplemental Figure 9C), but with a significant increase in rosette area (Figure 9A).

We observed an increased drying rate in both *agl22* mutants, suggesting increased water use (Figure 9B). To determine if this was due to developmental or metabolic changes we performed light response curve measurements of photosynthesis at specific times throughout the drying period (Supplemental Figure 9D). Due to the small leaf and rosette size, light curves were measured in whole plant chambers at 90%, 74% and 25% rSWC (see Methods). The increased water loss was primarily driven by a greater rosette area (Figure 9A), despite a significant reduction in stomatal conductance (Figure 9C). Accordingly, light saturated carbon assimilation (A_{sat}) was significantly reduced throughout the drying period already under well-watered conditions (Figure 9D), leading to a significant reduction in total aboveground biomass (Supplemental Figure 10A). Flowering time remained constant between well-watered and drought treatments in both *agl22* mutants,

492 indicating that drought stress conditions did not affect flowering time in the
493 *agl22* mutants (Figure 9E).
494
495 **Discussion**
496 **Chronology of the drought response suggests early adjustments in**
497 **stomatal conductance and carbon assimilation are followed by changes**
498 **in ABA and transcriptional reprogramming**
499 Responses to drought are complex and depend on the type and strength of
500 the drought stress imposed (Harb et al., 2010; Wilkins et al., 2010; Zhang et
501 al., 2014). The slow steady drought experiment carried out in this study
502 allowed us to investigate the full range of temporal physiological,
503 transcriptional and metabolic responses in a single fully expanded
504 Arabidopsis leaf. Additionally, by measuring leaf water potential (Figure 1B)
505 and RWC (Figure 1A) we were able to monitor the progression and degree of
506 drought stress in relation to the physiological, transcriptome and metabolome
507 changes. The decline in carbon assimilation at ~45% rSWC was primarily
508 driven by reduced stomatal conductance limiting CO_2 diffusion. There were no
509 underlying metabolic constraints, as photosynthetic capacity was unaffected
510 by the drought treatment (Figure 2, Supplemental Figure 1D), and metabolite
511 profiles remained unchanged throughout the majority of the drying period
512 (Figure 3A, Supplemental Figure 2 and Supplemental Figure 3), suggesting
513 that *Arabidopsis thaliana* Col-0 is a drought-tolerant ecotype.
514 Stomatal limitation as the primary factor in reducing photosynthesis under
515 mild drought conditions has been observed in other studies; however, severe
516 dehydration stress is believed to lead to metabolic constraints, associated
517 with RuBP availability (Flexas and Medrano, 2002). We did not observe a
518 reduction in the maximum capacity for carbon assimilation (A_{max}), rubisco
519 carboxylation (V_{Cmax}) and RuBP regeneration (J_{max}; Figure 2A). In addition
520 maximum and operating efficiencies of PSII photochemistry (Figure 2B),
521 photochemical- and non-photochemical quenching (Supplemental Figure 1D)
522 were maintained throughout the drying period despite a decline in carbon
523 assimilation (Figures 1D), indicating very little stress on photosystem II. This

524 suggested that an alternative electron sink, most likely photorespiration
525 (reviewed by Chaves et al., 2003; Lawson et al., 2014), must have been
526 operating under drought stress, and increased gene expression in the
527 photorespiratory pathway supports this notion (Figure 5C).
528 In general, two distinct phases in response to progressive soil drying could be
529 discerned (Figure 6 and Figure 7). Early responses were predominantly
530 adjustments to stomatal conductance leading to restricted CO_2 diffusion for
531 photosynthetic carbon assimilation (Boyer 1970; Passioura 1996; Figure 1C)
532 with some associated transcriptional changes accounting for 17 % of the 1815
533 DEGs (Supplemental Data Sets 1-12, Table 1). By contrast, late responses
534 (from 40% rSWC) encompassed hormonal (ABA), transcriptional and major
535 metabolic changes associated with senescence (Supplemental Data Sets 1-
536 12; Table 1). These later responses corresponded with the many different
537 phenological and physiological changes observed in other studies, including
538 impaired photosynthesis, increased solute accumulation and growth arrest
539 (Boyer 1970; Passioura 1996). At the cellular level, soluble sugars,
540 oligosaccharides, antioxidants and proline accumulation are known to
541 enhance the tolerance to drought stress by acting as osmolytes or as ROS
542 scavengers, especially hydroxyl radicals (Cuin and Shabala, 2007; Smirnoff
543 and Cumbes, 1989). The accumulation of these compounds during the latter
544 stages of the drought period (Figure 3B and Figure 3C), together with the
545 increase in secondary metabolites, such as flavonoids (Supplemental Data
546 Set 1, Supplemental Figure 3 and Supplemental Figure 4), suggests a role in
547 the defence against severe drought stress (Page et al 2012, Fini et al., 2011,
548 Tattini et al., 2004; Harb et al., 2010; Lei et al., 2006, Xiao et al., 2007, Xu et
549 al., 2008). In conclusion, this time series covers all phases during a
550 progressive drought stress, and therefore provides the opportunity to study
551 different stages of stress responses in greater detail than has been previously
552 possible (Supplemental Figure 10B).
553
554 **Transcriptional regulation of drought-stress responses**

555 At the gene expression level, a slowly developing soil water deficit is different
556 from rapid tissue dehydration. From the study here and in comparison with
557 two other drought experiments (Harb et al 2010, Wilkins et al 2010), it is clear
558 that different genes respond depending on the nature of the drought stress
559 applied (Figure 5A). Only few genes overlapped, with the majority of genes,
560 responding in the final 4 days of the experiment (Supplemental Data Set 8).
561 Previous studies have often focused on the identification of genes coding for
562 transcription factor classes responding to terminal or severe drought stress,
563 including BASIC LEUCINE ZIPPER (bZIPs, e.g. ABA responsive element
564 binding protein/ABRE binding factor), AP2/EREBP (e.g. DREB/CBF), NAC
565 transcription factors (NAM, ATAF1-2, CUPSHAPED COTYLEDON2), CCAAT-
566 binding (e.g. NUCLEAR FACTOR Y), and ZINC-FINGER (e.g. C2H2 zinc
567 finger protein) families (Licausi et al., 2010; Jensen et al., 2013; Li et al.,
568 2008; Karaba et al., 2007; Bartels and Sunkar, 2005; Umezawa et al., 2004).
569 The majority of TF genes in our study also responded relatively late in the
570 drought period (from 40% rSWC), especially those associated with ABA,
571 dehydration and oxidative stress responses (Supplemental Data Set 14).
572 Similar classes of TF genes have also been shown to respond to drought
573 stress in *Medicago truncatula* where 8% of the responding genes coding for
574 TFs responded late throughout the drying period (Zhang et al., 2014). By
575 contrast, among the early-responding TF genes (95% - 45% rSWC;
576 Supplemental Data Set 14), ~25% were linked to plant development,
577 indicating that early physiological changes may influence lifetime traits before
578 the initiation of acute stress defence and senescence responses, highlighting
579 the balancing act between the need to grow and to induce effective stress
580 tolerance mechanisms (Claeys and Inze, 2013).
581
582 **Dynamic Bayesian network modelling identifies genes that regulate**
583 **plant development**
584 Analysis of promoter binding sites (Supplemental Data Set 13, Supplemental
585 Figure 6), however, did not indicate specific regulatory networks or
586 mechanisms during the early events. We therefore assessed the use of a

high-throughput gene expression approach coupled with dynamic Bayesian network modelling to identify genes associated with the regulation of early drought responses. To make sense of large high-throughput datasets, network inference algorithms were developed, which are capable of establishing regulatory interactions among genes (Bansal et al., 2007). A number of different inference algorithms were used to successfully reconstruct known gene regulatory networks to validate these approaches (Cantone et al., 2009; Penfold and Wild, 2011). VBSSM is such an algorithm developed specifically for highly resolved temporal gene expression datasets, with the aim of identifying genes that are the key regulators in a given system (Beal et al., 2005). A recent comparison of modelling algorithms used to infer GRNs has shown that VBSSM is competitive with network reconstructions based on experimental data (Penfold and Wild, 2011; Windram et al., 2014; Penfold and Buchanan-Wollaston, 2014). Due to the limited number of experimental observations compared to the much greater number of differentially expressed genes, the system is inherently underdetermined (Penfold and Buchanan-Wollaston, 2014), and previous experience suggested that for these kinds of datasets VBSSM can model around 100 genes (Breeze et al., 2011). This type of approach, however, can introduce a bias during the process of gene selection, while the M-VBSSM approach (see Methods) generally avoids this gene selection bias (see Supplemental Methods). We therefore opted to use both M-VBSSM and VBSSM to identify key drought-regulatory genes and drought phenotypes associated with those genes.

The initial emergence of several development-associated transcription factors from the M-VBSSM approach (Supplemental Data Set 15) and the subsequent selection of developmental and non-developmental TFs for VBSSM (Supplemental Data Set 16) confirmed the flowering time regulator *AGL22* as a hub gene in the drought response. Importantly, we did not observe any involvement of *AGL22* in leaf senescence (Supplemental Data Set 9; Supplemental Figure 8A), suggesting that the gene plays a unique role in drought-stress responses.

The connection between flowering time and drought in Arabidopsis has been established independently in a number of studies, in which carbon isotope discrimination (Farquhar et al., 1982; 1989) QTLs co-located with known developmental/ flowering time loci (Hausmann et al., 2005; Masle et al., 2005; Juenger et al., 2005; McKay et al., 2008). The identification of a flowering time gene, and subsequent verification of its influence on plant water use, photosynthesis and phenology (discussed below), suggests that this type of dynamic modelling can provide an important means of discovering genes that will produce phenotypes associated with lifetime water use and plant development. However, unlike QTL mapping, VBSSM also managed to establish some valid network interactions from time-series transcriptomics data, potentially allowing for a temporal reconstruction of events and biological processes occurring during progressive drought stress.

A flowering time gene influences water use and photosynthesis under well-watered and drought-stress conditions

Both *agl22* mutants exhibited elevated water loss and rapid development already under non-stress conditions (Figure 9A, Figure 9B). This could imply a trade-off between drought-avoidance and -escape in environments where drought shortens the growing season (Franks, 2011). Selecting for early flowering may be beneficial for plant survival but not necessarily for achieving high biomass (Supplemental Figure 10A), which suggests that drought survival and the ability to maintain biomass under sustained water-limiting conditions depend on different mechanisms (Skirycz et al., 2011).

The *agl22* mutants exhibited 36% and 46% reductions in the steady state light- saturated photosynthetic rate under well-watered conditions (Figure 9D), which appeared to be partly associated with reduced stomatal conductance (Figure 9C). However, during drought stress the photosynthetic rate in both *agl22* mutants was reduced by only 11% and 13%, suggesting that both *agl22* mutants were able to maintain substantial photosynthetic rates (Figure 9D). This is supported by the fact that *agl22* mutants also maintained rosette growth throughout the drying period in comparison to wild-type plants (Figure

650 9A), and although total aboveground biomass was significantly reduced in
651 both mutant alleles (Supplemental Figure 10A), biomass distribution shifted
652 from vegetative growth to reproductive growth (Supplemental Figure 10C).
653 The complex links between plant growth, primary metabolism and flowering
654 time in Arabidopsis are highlighted in a recent paper where increased plant
655 growth was positively associated with early-flowering phenotypes (El-Lithy et
656 al 2010), which may explain the larger rosette area observed prior to flowering
657 in 30-day-old *agl22* mutant plants compared to wild type (Figure 9A;
658 Supplemental Figure 9B). In addition, starch/carbohydrate status and
659 metabolite levels have been linked to rosette growth (Meyer et al 2007;
660 Sulpice et al 2009), as well as development and flowering time (Zhou et al.,
661 1998; Moore et al., 2003; Funck et al., 2012). Both photosynthesis and
662 flowering are regulated by the light environment and are clearly linked via the
663 carbohydrate status (Zhou et al 1998; Moore et al 2003; Funck et al 2012),
664 connecting primary metabolism with plant growth and development. However
665 little is known about the link between photosynthetic performance and
666 flowering time especially in flowering time mutants, and at this point it is
667 unclear why the *agl22* mutants exhibited a substantial reduction in
668 photosynthesis already under well-watered conditions.
669 Furthermore, the observations regarding *AGL22* reinforces that water use in
670 relation to overall plant productivity requires a balance of developmental and
671 physiological processes to successfully complete a lifecycle in the prevailing
672 climatic conditions. *AGL22* was identified from moderately drought-stressed
673 plants, which also suggests that not all drought–responsive genes may work
674 in all water deficit scenarios. This may especially be the case for those genes
675 that have been selected under terminal or severe drought conditions (Hu et
676 al., 2006; Nelson et al., 2007; Xiao et al., 2009). It is important to note that
677 none of the TF genes selected as key regulators of the drought response in
678 earlier studies (Hu et al., 2006; Nelson et al., 2007; Xiao et al., 2009) was a
679 hub in the TF GRN (Figure 8A), although many of these TF genes were
680 differentially expressed during drought stress (Supplemental Data Set 14).
681 This is supported by the notion that many genes identified with a role in stress

682 tolerance under severe stress conditions seem to have little effect on plant
683 growth in mild drought conditions (Skirycz et al., 2011).

684 Interestingly, two targets of *AGL22*, *DREB1A* and *FBH3* (Figure 8D), have
685 previously been shown to be involved in the regulation of abiotic stress
686 responses. Over-expression of DREB1A leads to drought, salt and freezing
687 tolerance (Kasuga et al., 1999; 2004), while FBH3 has been shown to
688 regulate stomatal opening (Takahashi et al., 2013) and functions in ABA
689 signalling in response to osmotic stress (Yoshida et al., 2015). Both genes
690 were late-responding targets with few network connections (Figure 8A, and
691 Supplemental Data Set 15). The model therefore may allow us to predict the
692 role of AGL22 during drought stress and provide a potential link between
693 mild/moderate and severe drought responses.

694 This study demonstrates that network inference incorporating highly resolved
695 time-series transcriptomics data is able to predict TF networks and identify
696 genes with regulatory importance during drought stress. Moreover, by
697 focusing on the transition from early physiological changes to drought stress
698 responses we were able to identify *AGL22* as a gene associated with lifetime
699 water use. Consequently, VBSSM as a gene discovery tool promotes the
700 selection of unknown, yet highly connected genes for further phenotypic
701 evaluation.

702

703 **METHODS**

704 **Plant material, plant growth and drought stress**

705 *Arabidopsis thaliana* plants (Col-0, *agl22*-3 [SALK_141674], and *agl22*-4
706 [SAIL_583_C08]) were obtained from the European Arabidopsis Stock
707 Centre, and were grown under a 8:16-h light:dark cycle at 23°C, 60% relative
708 humidity, and light intensity of 150 µmol m^{-2} s^{-1}, using a mixture of cold and
709 warm white fluorescent tubes. Arabidopsis seed was stratified for 3 d in 0.1%
710 agarose at 4°C before individual seeds were sown onto a soil mix (Scotts
711 Levingtons F2+S compost : fine grade vermiculite in a ratio of 6:1). For the
712 drought time-course experiment, pots (7cm x 7cm x 9 cm) were filled with the

same amount of soil mix. Control pots, to determine 100% and 0% soil water content, were set up at the same time. Plants were transferred into individual pots 2 weeks after the sowing date and were kept well watered until the beginning of the drying episode at 5 weeks after sowing. Half the plants were maintained under well-watered conditions, while for the remaining half, water was withdrawn and pot weight was determined daily. Relative soil water content was calculated for each day and pots were left to dry until 17% rSWC was reached. Five-week-old plants were saturated in water to reach 95% rSWC, and watering was stopped in the treatment plants until ~17% rSWC was reached. The control plants were maintained under well-watered conditions at ~95% rSWC. Due to the early-flowering phenotype of both *agl22* mutant alleles, drought experiments were carried out on 22-day-old plants to ensure the experiments were carried out at similar rosette developmental stages and prior to the onset of flowering, as indicated in Supplemental Figure 9B and C. The drying rate was determined as the slope of the decline in relative soil water content, measured daily throughout the drying period.

RNA extractions, labelling, microarray hybridisation and analysis

Total RNA was extracted, labeled, and hybridized to CATMA v4 arrays (Sclep et al., 2007) as previously described (Breeze et al., 2011; Windram et al., 2012). The experimental design for the drought time-series hybridization is shown in Supplemental Figure 11.

Arrays were hybridized and washed as described in (Windram et al 2012). Arrays were scanned on a 428 Affymetrix scanner at wavelengths of 532 nm for Cy3 and 635 nm for Cy5. Cy3 and Cy5 scans for each slide were combined and processed in ImaGene version 8.0 (BioDiscovery) to extract raw intensity and background corrected data values for each spot on the array. The data have been deposited in Gene Expression Omnibus under the accession number GSE65046. URL for review: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cfgvmssmnbylpsr&acc=GSE65046

744 An adaptation of the MAANOVA package (Wu et al., 2003) was used to
745 analyze the extracted microarray data as described by Breeze et al. (2011)
746 and Windram et al. (2012), using a mixed-model analysis. The MAANOVA
747 fitted model considered dye and array slide as random variables, and time
748 point, treatment and biological replicate as fixed variables. The model allowed
749 assessment of the main effect of treatment, the main effect of time point, the
750 interaction between these factors, and the nested effect of biological replicate.
751 Predicted means were calculated for each gene in each of the 112
752 combinations of treatment, time point and biological replicate, and for each of
753 the 28 combinations of treatment and time point, averaged across biological
754 replicates.
755
756 **Differential gene expression analysis**
757 Genes were ranked based on their Gaussian process two-sample (GP2S)
758 Bayes Factor DE score, a cutoff of ≥5 gave 2496 differentially expressed
759 genes. Genes identified in the F-Test as being differentially expressed that
760 were not in the GP2S list of 2496 genes were added manually. The
761 expression profiles of the genes ranked 1800-3150 were then plotted and
762 assigned visually as DE or not. This resulted in a false positive rate of 23.3%
763 for this group, and a final cutoff of 6 for the GP2S was chosen, duplicates
764 were removed, and a list of 1934 differentially expressed genes was
765 produced. Removal of probes and genes with no annotation in TAIR9 left a list
766 of 1815 unique differentially expressed genes. The time at which genes first
767 became differentially expressed (TOFDE) was subsequently determined using
768 the GP2S time-local method (Stegle et al., 2010).
769
770 **Promoter Analysis**
771 Publically available position-specific scoring matrices (PSSMs) were collected
772 from the PLACE and JASPAR databases (Higo et al., 1999, Sandelin et al
773 2004). PSSMs were clustered by similarity, and a representative of each
774 cluster was chosen for screening. Promoter regions corresponding to 200 bp
775 upstream of the transcription start site were retrieved from the Ensembl Plants

776 sequence database (release 50). For any given PSSM and promoter, we
777 scanned the sequence and computed a matrix similarity score (Kel et al.,
778 2003) at each position on both strands. P values for each score were
779 computed from a score distribution obtained by applying the PSSM to
780 randomly generated sequences. We took the top *k* non-overlapping hits and
781 performed the binomial test (pbinom function in R Stats package) for the
782 occurrence of *k* sites with observed p-values within a sequence of length 200
783 bp. The parameter *k* is optimized within the range 1 to 5 for minimum binomial
784 P-value to allow detection of binding sites without a fixed threshold per
785 binding site. To determine the presence or absence of a PSSM in a promoter,
786 in each case the promoters were sorted by binomial p-value, and we applied a
787 cutoff to select the top 2000. For each PSSM, its frequency in promoters of
788 each cluster was compared with its occurrence in all promoters in the
789 genome. Motif enrichment was calculated using the hypergeometric
790 distribution (see statistical analysis). For motif enrichment analyses p-values ≤
791 1e-5 were considered significant, to allow for multiple testing.
792
793 **Reverse transcription (RT-PCR) and quantitative real time PCR (qPCR)**
794 Leaves were harvested and frozen in liquid nitrogen. Total RNA was extracted
795 from a pool of 3 plants using Tri-reagent (SIGMA, Aldrich, UK) according to
796 the manufacturer's instructions. A minimum of five replicates of whole plants
797 from separate experiments was carried out for mutant analysis and drought
798 treatments. cDNA synthesis for quantitative real time PCR (qPCR) and
799 reverse transcriptase PCR (RT-PCR), 1 µg total RNA was treated with
800 RNase-free DNase (Ambion) according to manufacturer's instructions and
801 reverse transcribed as previously described (Ball et al., 2004). qPCR-PCR
802 was performed using a SYBR green fluorescence based assay as described
803 previously (Bechtold et al., 2010; 2013). Gene-specific cDNA amounts were
804 calculated from threshold cycle (Ct) values and expressed relative to controls
805 and normalized with respect to *ACTIN* and *CYCLOPHILIN* cDNA according to
806 Gruber et al. (2001). RT-PCR was carried out to amplify the full-length *AGL22*

gene on both *agl22* mutant alleles. The primers used for qPCR and RT-PCR are given in Supplemental Table 1.

Physiological measurements

(i) Relative water content

Whole rosettes of 5 plants were harvested each day throughout the drying period. The relative water content (RWC) of the leaf was calculated using the formula: rLWC (%) = (FW-DW)/ (SW-DW) x 100, where FW is the actual rosette weight at the day of harvest, SW is the fully saturated rosette weight, and DW is the dry weight of the rosette.

(ii) Leaf water potential

The leaf water potential was measured via the Scholander pressure bomb technique (Scholander et al., 1964) using a Plant Moisture System SKPM1400 (Skye Instruments Ltd, Powys, UK). Leaf water potential was measured daily throughout the drying period on both control and drought-stressed plants according to the manufacturer's instructions.

(iii) Plant development and biomass measurements

Arabidopsis development was assessed using the scale developed by Boyes et al. (2001). Once the final flower had opened, watering was ceased and plants were bagged and left to dry out before harvesting. At harvesting, rosettes, stalks and seeds were separated. The seed weight, dry weight of rosettes and stalks/pods were determined (Bechtold et al., 2010). At least 10 plants per line and watering regime were measured.

Photosynthesis measurements

(i) Photosynthetic rate (snapshot measurements)

Instantaneous measurements of net CO_2 uptake rate (*A*) and stomatal conductance to water (*gs*) were made on leaf 7, using an open gas exchange system (PP Systems, Amesbury, MA, USA). Leaves were placed in the cuvette at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1}, leaf temperature

was maintained at 22 ± 2 °C and vapour pressure deficit was *ca.* 1 kPa and irradiance was set to growth conditions (150 µmol m^{-2} s^{-1}). A reading was recorded every 3 minutes when the IRGA conditions had stabilized (*ca.* 1.5 min), but before the leaf had a response to the new environment (Parsons et al., 1997).

(ii) A/Ci curves (maximum photosynthetic rates)

Five weeks after emergence, (*A*) and (*gs*) was measured on leaf 7, using an infrared gas exchange system (PP Systems, Amesbury, MA, USA). The response of *A* to changes in the intercellular CO_2 concentration (c_i) was measured under a saturating photon flux density (PFD), provided by a combination of red and white LEDs (PP Systems, Amesbury, MA, USA). In addition, the response of *A* to changes in PFD from saturating to sub-saturating levels was measured using the same light source at the current atmospheric CO_2 concentration (390 µmol mol^{-1}). All gas analysis was made at a leaf temperature of 20 (±1) °C and a vapour pressure deficit of 1 (± 0.2) KPa. Plants were sampled between 1 and 4 hours after the beginning of the photoperiod. For each leaf, steady state rates of *A* and g_s at current atmospheric [CO_2] were recorded at the beginning of each measurement. The A/c_i parameters, V_{Cmax} (maximum RubP-saturated rate of carboxylation *in vivo), A_{max} (light* and CO_2 saturated rate of carbon assimilation *in vivo*) and J_{max} (maximum *in vivo* rate of electron transport contributing to *RuBP* regeneration) were calculated by fitting equations described in (Farquhar et al., 1980) with subsequent modifications described in (McMurtrie and Wang, 1993).

(iii) Chlorophyll fluorescence imaging

Plants were analysed at various stages of the progressive drought stress using the dark (*Fv/Fm*)- and light (*Fv'/Fm'*, *Fq'/Fm'* and *Fq'/Fv'*)-adapted chlorophyll *a* fluorescence parameters, using a chlorophyll fluorescence imaging instrument (Fluorimager, Technologica, Colchester UK; Barbagallo et al., 2003).

(iv) Light response curves using whole plant chambers

A/Q response curves were measured using whole-plant gas exchange system developed at the University of Essex, with a heliospectra LED light source (Heliospectra AB, Göteborg, Sweden). Input air was maintained at a relative humidity of 50-60%, air temperature of 22°C, and CO_2 concentration of 400 mmol mol^{-1}, matching that of the growth conditions. Plants were initially stabilized for 30 minutes at saturating irradiance 800 µmol m^{-2} s^{-1}, after which PPFD was reduced in 9 steps (Supplemental Figure 9D), with assimilation (*A*) and stomatal conductance (g_s) being recorded at each new PPFD level.

Metabolite and hormone analysis

During the experimental period, 2 leaves per plant were harvested every day, in total 6 plants per treatment. Samples were frozen in liquid nitrogen, freeze dried overnight and stored at room temperature in darkness until extraction. Primary metabolites were extracted from frozen tissue with chloroform-methanol as described in Lunn et al. (2006). T6P, other phosphorylated intermediates and organic acids were measured by high performance anion-exchange chromatography coupled to tandem mass spectrometry as described in Lunn et al. (2006). Trehalose was measured enzymatically with fluorometric detection as described in Carillo et al. (2013).

For sugars, amino acids, hormones and secondary metabolites, freeze dried leaf powder (10 mg) was extracted in 0.8 mL methanol containing 1% acetic acid. After centrifugation (10 min at 16 100 *g*, 4 °C), the samples were filtered through a 0.2 µm PVDF syringe filter (Chromacol). For non-targeted LC-QToF MS metabolite profiling 5 µL extract was injected onto a Zorbax StableBond C18 1.8 mm, 2.1 x 100 mm (QTOF) reversed-phase analytical column (Agilent Technologies). Chromatography and MS conditions are described by Page et al (2012). Peaks were extracted and aligned using XCMS (Smith et al., 2006) and statistical analysis and data visualisation were carried out with MetaboAnalyst 2.0 (Xia et al., 2012). For LC–MS/MS analysis of hormones, 10 mg freeze-dried leaf powder was extracted in 0.8 mL 10% methanol + 1%

903 acetic acid containing deuterated standards (Forcat et al., 2008). Secondary
904 metabolites and hormones were analysed with an Agilent 6420B triple
905 quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, CA,
906 USA) coupled to a 1200 series Rapid Resolution HPLC system. 2 µL, of
907 sample extract was loaded onto a Zorbax Eclipse Plus C18 3.5 mm, 2.1 x 150
908 mm for amino acids, sugars and hormones, respectively. The following
909 gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min –
910 100% B; 25 min – 100% B; 27 min – 0% B; 7 min post-time. QQQ source
911 conditions were as follows: gas temperature 350 °C, drying gas flow rate 9 L
912 min^{-1}, nebulizer pressure 35 psig, capillary voltage 4 kV. The polarity,
913 fragmentor voltage and collision energies were optimized for each compound.
914 The MRMs used for compound identification are shown in Supplemental Data
915 Set 17, and data are reported as peak areas. Flavonoid identification was
916 based on previous MS/MS identification of flavonoids in *Arabidopsis* (Tohge et
917 al., 2005; Stobiecki et al., 2006). For sugar and polyol analysis, 5 µL sample
918 extract was loaded onto an XBridge^{TM} amide HILIC column (particle size 3.5
919 µm, 2.1 mm ID x 150 mm, Waters, UK) with a constant flow rate of 0.3 mL
920 min^{-1} and a column temperature of 35^{o}C for the duration. Mobile phases
921 comprised of water:acetonitrile with 0.1% ammonia (mobile phase A was 90%
922 acetonitrile, and B was 10% acetonitrile with 5 mM ammonium formate).
923 Sugars (5 µL) were separated using the following gradient: 0-17 min, 0-54%
924 B; 17-19 min, 54% B; 19-20 min, 54-0% B, with a 10 min re-equilibration time.
925 The QQQ was operated in negative ion mode. ESI source conditions were
926 gas temperature 350°C, drying gas flow rate 9 L min^{-1}, nebuliser pressure 35
927 psig, and capillary voltage 4 kV. Data were acquired in selected ion
928 monitoring (SIM) mode with a dwell time of 50 msec. The fragmentor voltage
929 was 50 V for all sugars. The sugars were quantified by reference to standards
930 (Supplemental Data Set 17). Amino acids were separated with a ZIC-HILIC
931 column (150 x 2.1 cm, 3.5 µm particle size; Merck SeQuant, Sweden).
932 Sample (2 µl) was injected into the column with a flow rate of 0.25 mL min^{-1}.
933 Mobile phases comprised of water: acetonitrile with 0.1% formic acid (mobile
934 phase A was 95% acetonitrile and B was 5% acetonitrile with 5 mM

ammonium acetate). Compounds were separated using the following gradient: 0-10 min, 5-50% B, 10-15 min, 50-90% B, 15-20 min, 90% B, 20-25 min, 90-5% B, with an 11 min re-equilibration time. The QQQ was operated in positive ion mode and ESI source conditions were gas temperature 350°C, drying gas flow rate 9 L min^{-1}, nebuliser pressure 35 psig, and capillary voltage 4 kV. Data were acquired in multiple reaction monitoring (MRM) mode with a dwell time of 50 msec. Amino acids were quantified by MRM (Supplemental Data Set 17) and data are reported as peak areas (Supplemental Data Set 18).

Analysis of publicly available microarray datasets

Publicly available microarray datasets from different experiments in which Arabidopsis was subjected to drought and senescence (Wilkins et al., 2010; Harb et al., 2010; Breeze et al 2011) were located in supplementary files of already published papers, and were compared with the drought timeseries datasets using VENNY (Oliveros, 2007). Hypergeometric distributions were calculated for different overlaps using the phyper function in R version 3.0.2. A cutoff of -p(log) of 5 was chosen as highly a significant overlap between two or more datasets.

Analysis of Gene Ontology (GO)

GO annotation analysis was performed using Database for Annotation, Visualization and Integrated Discovery v6.7 (DAVID; Huang et al., 2008), BINGO (Maere et al., 2005) and Agrigo (Du et al., 2010) with the GO_Biological_Process category, as described by Ashburner et al. (2000). Overrepresented GO_Biological_Process and GO_Molecular_Function categories were identified using a hypergeometric test with a significance threshold of 0.05 after Benjamini-Hochberg correction (Benjamini and Hochberg, 1995) or Bonferroni correction (Holm, 1979) with the whole annotated genome as the reference set.

Variational State Space Modelling (VBSSM) and Metropolis - VBSSM

Significant numbers of genes can be differentially expressed (DE) in response to environmental stress, which, given the limited number of experimental measurements, means that network models are often unidentifiable (see e.g., Penfold and Buchanan-Wollaston, 2014; Windram et al., 2014 and references therein). Furthermore, the interpretation of large, densely connected networks can often be difficult, and any hypothesis we extract from them can therefore be ambiguous. One solution is to select a more limited number of genes to model, either based upon prior knowledge, heuristic approaches or random selection. The reduced number of genes means network inference approaches can be applied such as the Variational Bayesian State Space modeling (VBSSM) of Beal et al. (2005). Within the VBSSM the expression of the genes can be written in the form:

$$\boldsymbol{y}_t \propto [\boldsymbol{BC} + \boldsymbol{D}]\boldsymbol{y}_{t-1},$$

where the term $[\boldsymbol{BC} + \boldsymbol{D}]_{ij}$ captures all information about how gene *j* regulates gene *i*. Rather than infer a point estimate for each interaction $[\boldsymbol{BC} + \boldsymbol{D}]_{ij}$ Beal et al. (2005) infers a posterior distribution, and use standard Z-statistics to assess the statistical significance. The pre-selection of genes described above may, however, result in some bias. Here we chose to additionally build a network model around a particular gene of interest, using random selection via a Metropolis algorithm. At each step in the Metropolis algorithm a Bayesian state space model is fitted to the time-series gene expression profiles for the selected genes, and the marginal likelihood or "model evidence" used as the selection criteria. In this way we can infer small network models around each gene that we are interested in (for full details see Supplemental Methods, Supplemental Figure 12).

For the drought data, a total of 176 transcription factors were differentially expressed (Supplemental Data Set 14). We therefore use the Metropolis model selection to systematically build a network of 88 genes around each of the 176 genes in turn. Within the Metropolis selection each of the 176 network models were run for 2000 iterations in the MCMC chain, by which point the Marginal Likelihood was seen to be plateau and the algorithm was terminated.

The 176 networks at step 2000 were then combined to create a meta-network, which was used to compile summary statistics; such as the number of times a particular gene was found in each of those 176 network models, or the number of downstream connections a particular gene had over those 176 models. Ranked lists of genes can be found in Supplemental Data Set 13. Of the top 10 genes with the highest number of downstream connections, 4 were annotated as being developmental in nature, suggesting a link between drought response and developmental programs. A selection of 99 random differentially expressed transcription factors including the top 10 highly connected genes were selected from the larger pool of 176 TFs that were DE during the drought stress (Supplemental Data Set 16). Both the control and drought time series for these genes were normalised to have zero-mean and unit variance and subsequently modelled using the VBSSM of Beal *et al*. (2005) using a z-score of 1.65 to select the control and drought-specific networks, and a final VBSSM model (Beal et al., 2005) was fitted to the gene expression for *AGL22*.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (Chicago, IL, USA; http://www.spss.com/). Parameter differences between wild type and *agl22* mutants were determined using one-way analysis of variance (ANOVA) with appropriate post-hoc analysis. TukeyHSD test was used if variances of means were homogenous, and Games Howell test, if variances were not homogenous. The standard error of the calculated ratios of fold differences for metabolite and gene expression data, errors of individual means were combined "in quadrature" as the final ratio was a combination of the error of the two different means of the control and drought stress samples. Correlations were estimated among drying rate and flowering time as the standard Pearson product-moment correlation between the genotype means. Hypergeometric distributions were analysed using the phyper function in the R stats package. Generally, p-values ≤ 1e-5 were considered significant, to allow for multiple testing. The metabolite data were analysed by between

subjects 2-way ANOVA for time series data and probability values with false discovery rate multiple testing correction are tabulated. For secondary compounds, amino acids and sugars, compounds not detected in >50% of samples were discarded and remaining missing data imputed using KNN (see Supplemental Data Set 3). Abundance data were normalised to total signal in each sample, log2 transformed, mean-centred and divided by standard deviation of each variable using Metabolonalyst 3.0 (Xia et al., 2015).

Accession numbers

Sequence data from this article can be found in the Gene Expression Omnibus data libraries under accession number GSE65046

Supplemental Data

The following materials are available in the online version of this article

Supplemental Figure 1: Plant growth and chlorophyll fluorescence during progressive drought stress.

Supplemental Figure 2: Targeted metabolite analyses of secondary metabolites (except flavonoids), sugars and amino acids.

Supplemental Figure 3: Targeted metabolite analyses of flavonoids and anthocyanins.

Supplemental Figure 4: The effect of drought stress on plant development.

Supplemental Figure 5: Temporal expression patterns of five selected flavonol biosynthesis genes.

Supplemental Figure 6: Analysis of TF binding sites.

Supplemental Figure 7: Gene expression of selected drought responsive genes and growth analysis of *agl22* mutants.

Supplemental Figure 8: Validation of the knockout phenotype in *agl22* insertion mutants and the specific role of *AGL22* during drought stress.

Supplemental Figure 9: Growth and photosynthetic phenotype of Col-0 and *agl22* mutants during drought stress

Supplemental Figure 10: Biomass production in *agl22* mutants compared to wild type and timeline of events.

Supplemental Figure 11: Schematic overview of array hybridizations across the 13 time points and two different treatments.

Supplemental Figure 12: Validation of the M-VBSSM approach

Supplemental Table 1: Primers for qPCR, mutant screen and RT-PCR analyses

Supplemental Methods: Model Comparison via a Metropolis Search

Supplemental Data Set 1: LC-MS of sugars and LC-MS/MS analysis of secondary metabolites and amino acids

Supplemental Data Set 2: LC-MS/MS analysis of sugar phosphates, other phosphorylated compounds, and organic acids.

Supplemental Data Set 3: ANOVA of metabolites

Supplemental Data Set 4: List of differentially expressed genes

Supplemental Data Set 5: Functional categorisation of 1815 DEG

Supplemental Data Set 6: Time of first differential expression

Supplemental Data Set 7: Differentially expressed genes from publicly available drought and senescence datasets

Supplemental Data Set 8: Comparison with published drought datasets

Supplemental Data Set 9: Comparison with a published senescence dataset

Supplemental Data Set 10: Gene ontology analysis of genes overlapping in the drought and senescence time series.

Supplemental Data Set 11: Hierarchical Clustering divides the 1815 genes into 28 Clusters

Supplemental Data Set 12: Gene ontology analysis; over- and under-representation of Biological Process (BP) and Molecular Function (MF) of individual clusters

Supplemental Data Set 13: Analysis of TF binding sites

Supplemental Data Set 14: Differentially expressed transcription regulators divided into transcription factor families

Supplemental Data Set 15: Output of the TF Metropolis VBSSM (M-VBSSM)

Supplemental Data Set 16: Selected TFs for VBSSM modelling

Supplemental Data Set 17: MRMs used for compound identification

Supplemental Data Set 18: Output from xcms alignment of peaks from LC-QToF metabolite profiling

Author contributions

U.B. designed, carried out and analyzed the drought experiments. TL designed the gas exchange and fluorescence measurements. J.S.A.M. and S.R.M.V-C performed whole-chamber gas exchange experiments on gene mutants. S.S. isolated hub gene mutants. R.F. and J.E.L. measured primary metabolites. H.F., C.S., D.L.S. and N.S. measured and analyzed targeted and untargeted metabolites. C.A.P., D.J.J., L.B., L.B., R.H., S.O., R.L., C.H., J.D.M. were responsible for data analysis. K.J.D, N.R.B., P.M.M., N.S., A.M., D.L.W., B.F., D.R., J.B., S.O., V.B.-W., and U.B. all had input into the design of the experiments and analysis. U.B. wrote the article with contributions from all authors.

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Figure legends:

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Figure 2: Potential photosynthesis in response to drought. A - A/C_i curves were performed under saturating light conditions of 1000 µmol m^{-2} s^{-1} at six selected time points throughout the drying period, and potential photosynthesis was calculated including: light and [CO_2]-saturated net CO_2 assimilation (A_{max}); maximum rate of RubP regeneration (J_{max}), and maximum rate of carboxylation ($Vc_{,max}$), and **B** - maximum and operating quantum efficiencies of photosystem II (Fv/Fm, Fq'/Fm' and Fv'/Fm'). The data represent the mean (n= 3; ± s.e.m.).

Figure 3: Metabolite levels during progressive drought. A - LC-ESI-QToF MS metabolite profiling of Arabidopsis leaves under well-watered (W) and progressive soil drought (D) conditions. Leaf extracts were analysed in negative and positive ionisation modes. The heat maps show the normalised abundances of all detected chemical features. Samples and chemical features were clustered using a Pearson distance measure and the Ward clustering algorithm (see Supplemental Data Set 18); **B** – relative concentrations of ABA (black bars) and proline (grey bars), and **C** - relative concentrations of galactinol (black bars) and raffinose (grey bars). The data represent the mean of the ratio (n=4; ± s.e.m; * $p < 0.05$, or ** $p < 0.01$).

Figure 4: The relationship between stress severity and differential gene expression. A – number of genes for which the first differential expression was observed at each time point, indicating a late transcriptional response; **B** – correlation between leaf water potential and rSWC; **C** – correlation between leaf water potential and number of differentially expressed genes, and **D** – correlation between carbon assimilation and leaf water potential. Line represents the linear regression, r^2 and p values are given.

Figure 5: Comparative meta-analysis with publicly available datasets, and MapMan analysis of primary and secondary metabolism pathways. A - Comparative meta-analysis of the 1815 DEGs with publicly available drought datasets. The Venn diagram shows the overlap of time series DEGs with those responsive to moderate (mDr; Harb et al., 2010) or progressive drought (pDr; Harb et al., 2010), and a moderate drought at different times of day (diel, Wilkins et al., 2010); **B** - Comparative meta-analysis of the 1815 DEGs with a publicly available leaf 7 senescence time-series dataset (Breeze et al., 2011). The Venn diagram shows the overlap of drought - and senescence DEGs; **C** - overview of antioxidant, photosynthesis and photorespiration-related gene expression at two different time points (95% rSWC and 17% rSWC); **D** – overview of oxidation/reduction-related gene expression at two different time points (95% rSWC and 17% rSWC), and **E** – overview of secondary metabolism-related gene expression at two different time points (95% rSWC and 17% rSWC). All MapMan diagrams show gene expression data in leaf 7, where blue indicates increased and yellow indicates decreased gene expression according to the scale. Each square represents a single gene within the pathways.

Figure 6: Temporal clustering of 1815 differentially expressed genes. A - Dendrogram of the hierarchical clustering using a Euclidian distance divides the dataset into early and late responses for both the control (white circles) and drought (grey circles) samples, and **B** - heat map of the SplineCluster analysis of the 1815 DEGs on differentially expressed genes (drought samples only) across the time series using normalised and averaged data (Supplemental Data Set 11). The heat map demonstrates expression profiles for genes in each cluster with red representing high expression and green representing low expression.

Figure 7: Descriptions of the 28 clusters derived from SplineCluster. The blue line indicates the mean expression profile for each of the 28 clusters. Individual genes present in each cluster are available in Supplemental Data Set 11. The red line indicates the switch from early (95% - 45% rSW; days 1-7) to late (40% - 17% rSWC; days 8 - 13). Selected enriched GO terms (see Supplemental Data Set 12) are indicated on each cluster.

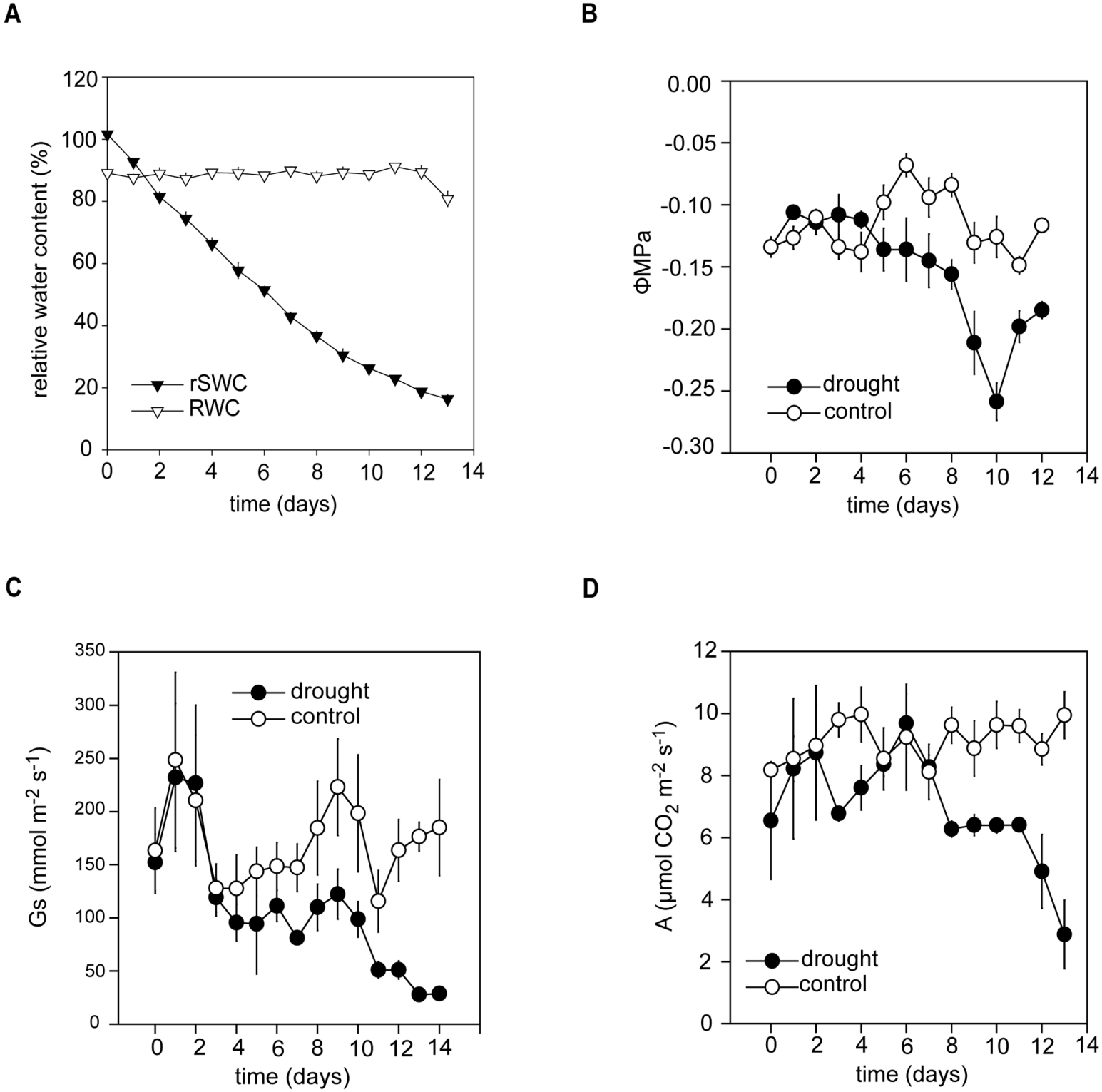
Figure 8: Constructing and evaluating a transcription factor regulatory

network. A - gene regulatory network generated using VBSSM with the drought time-series data (threshold z-score = 1.65). The nodes highlighted in red were up-regulated during drought stress including the central hub gene, *AGL22*. Nodes highlighted in green were genes down regulated during drought stress. Blue nodes signify genes that were not regulated by *AGL22* as predicted from the model (see panel D). All red, green and blue nodes were selected for evaluation after drought stress; **B** - relative gene expression of selected genes under drought conditions (17% rSWC). Gene expression was analysed by qPCR. The numbers are expressed as fold changes of drought over control (n=5 ± s.e.m). Significance of the fold changes are indicated by either * $p < 0.05$, or ** $p < 0.01$. For gene and primer list see Supplemental Data Set 16 and Supplemental Table 1; **C** - relative expression levels of *AGL22* in two knockout lines, *agl22-3* and *agl22-4,* compared to wild-type determined by qPCR. Significance of the fold changes are indicated ** $p < 0.01$, and **D** – relative gene expression profiles of 16 genes predicted to be regulated by *AGL22* under drought stress in *agl22-3* (black bars) and *agl22*-4 (grey bars) compared to wild type. The data represent the mean (n =7; ± s.e.m.), significance of the fold changes are indicated by either * $p < 0.05$, or ** $p < 0.01$. Stars located centrally indicate both mutants are significantly different to wild type, while stars located over one mutant indicate significance for the specific mutant.

Figure 9: Stress and plant growth phenotypes of *agl22* mutants. Due to the early-flowering phenotype of both *agl22* mutant alleles, drought stress was begun at 22-days after sowing. **A** - rosette area (cm^2) of Col-0 (light grey), *agl22-3* (black) and *agl22-4* (dark grey) plants at different soil water contents (n=5). The * indicates significant difference compared to wild type at $p < 0.05$; **B** - rate of water loss in *agl22-3* and *agl22-4* plants compared to wild type averaged over 13 days water withdrawal (n = 10). The * indicates significant difference compared to wild-type at $p < 0.05$; **C** - stomatal conductance (Gs) at different soil water contents in Co-0 (light grey), *agl22-3* (black) and *agl22-4* (dark grey), (n=5). The * indicates significant difference compared to wild-type at $p < 0.05$; **D** – light saturated carbon assimilation (A*sat*) at different soil water contents in Col-0 (light grey), *agl22*-3 (black) and *agl22*-4 (dark grey; n=5). The * indicates significant difference compared to wild type at $p < 0.05$, and **E** - days to flowering in well-watered (light grey) and drought-stressed (black) plants (n=10). Plants were grown under short day conditions as described in the Methods section. At 5 weeks, plants were subjected to progressive drought stress. When 17% rSWC was reached, plants were re-watered and flowering time was recorded as days after sowing. Control plants were maintained well watered. The * indicates significant difference compared to wild type at $p < 0.05$.

Table 1: Functional categorisation of early (95- 45% rSWC) and late (40% - 17% rSWC) responsive genes. P-value, adjusted using the Benajmini-Hochberg method (Benjamini and Hochberg, 1995). Category - indicates the timing and direction of change in gene expression across the time series. Fold - indicates the fold enrichment.

Category	GO term	Biological Process/ Molecular Function	Fold	P-value
early up-regulated	GO:0034637	cellular carbohydrate biosynthetic process	6.9	0.003
early up-regulated	GO:0006812	cation transport	4.3	0.014
early up-regulated	GO:0044262	cellular carbohydrate metabolic process	3.7	0.018
early up-regulated	GO:0022890	inorganic cation transmembrane transporter activity	5.1	0.0432
late up-regulated	GO:0009812	flavonoid metabolic process	7.1	1.74E-05
late up-regulated	GO:0009813	flavonoid biosynthetic process	7.1	5.82E-05
late up-regulated	GO:0019748	secondary metabolic process	2.3	0.004
late up-regulated	GO:0009699	phenylpropanoid biosynthetic process	3.8	0.006
late up-regulated	GO:0016051	carbohydrate biosynthetic process	2.7	0.011
late up-regulated	GO:0006519	cellular amino acid and derivative metabolic process	2	0.011
late up-regulated	GO:0019438	aromatic compound biosynthetic process	2.9	0.021
late up-regulated	GO:0042398	cellular amino acid derivative biosynthetic process	2.9	0.044
late down-regulated	GO:0006412	translation	2.1	4.96E-05
late down-regulated	GO:0015995	chlorophyll biosynthetic process	11.4	6.50E-04
late down-regulated	GO:0044085	cellular component biogenesis	2.4	0.002
late down-regulated	GO:0042254	ribosome biogenesis	3.6	0.003
late down-regulated	GO:0006334	nucleosome assembly	6.9	0.003
late down-regulated	GO:0015979	photosynthesis	3.9	0.011
late down-regulated	GO:0033014	tetrapyrrole biosynthetic process	7.7	0.014
late down-regulated	GO:0055114	oxidation reduction	1.8	0.02



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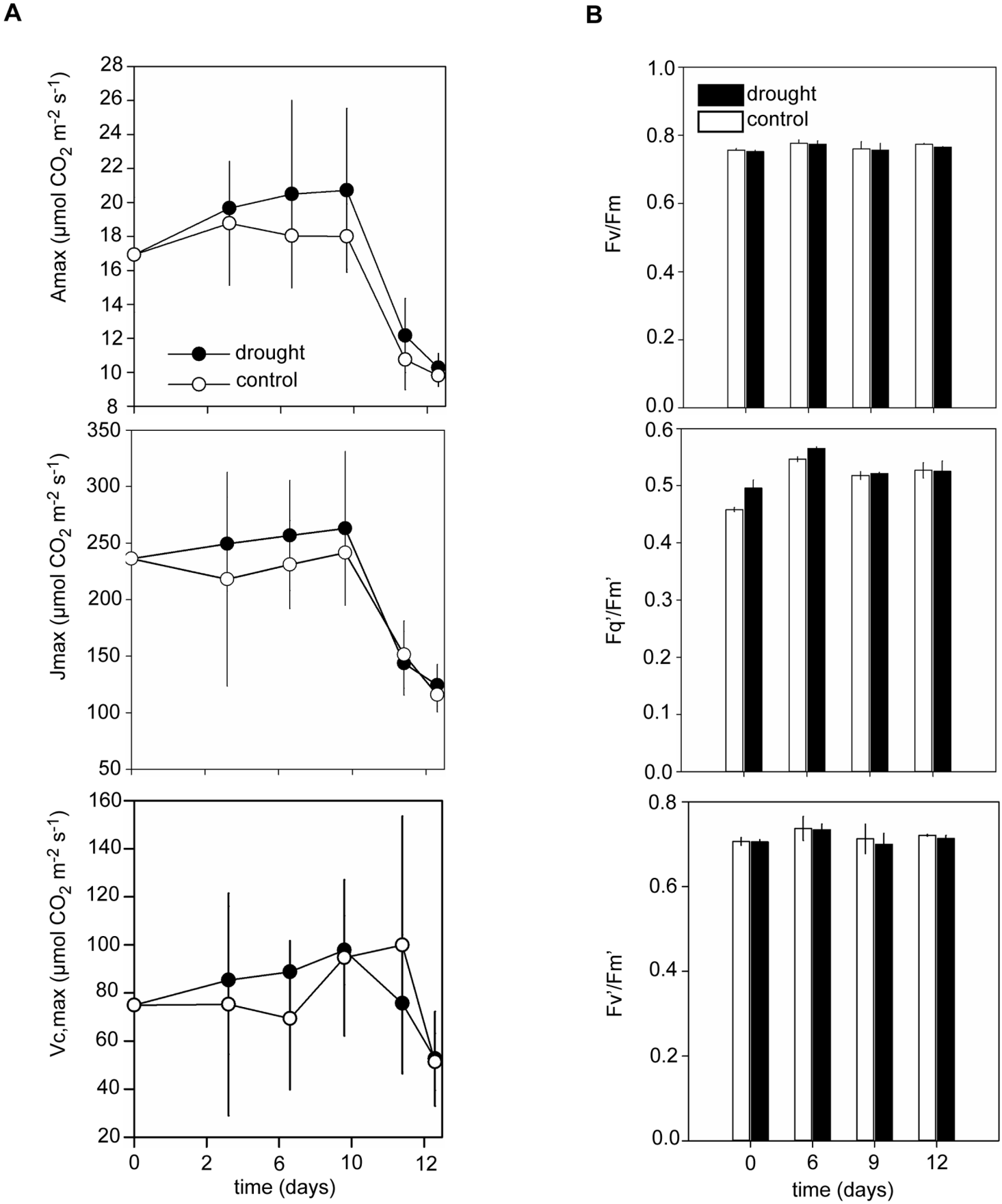

Figure 2: Potential photosynthesis in response to drought. A - A/Ci curves were performed at saturating light conditions of 1000 µmol m^{-2} s^{-1} at six selected time points throughout the drying period, and potential photosynthesis was calculated including: light and [CO_2] saturated net CO_2 assimilation (Amax); maximum rate of RubP regeneration (Jmax), and maximum rate of carboxylation (Vc,max), and **B** - maximum and operating quantum efficiencies of photosystem II (Fv/Fm, Fq'/Fm' and Fv'/Fm'). The data represent the mean (n= 3; ± s.e.m.).

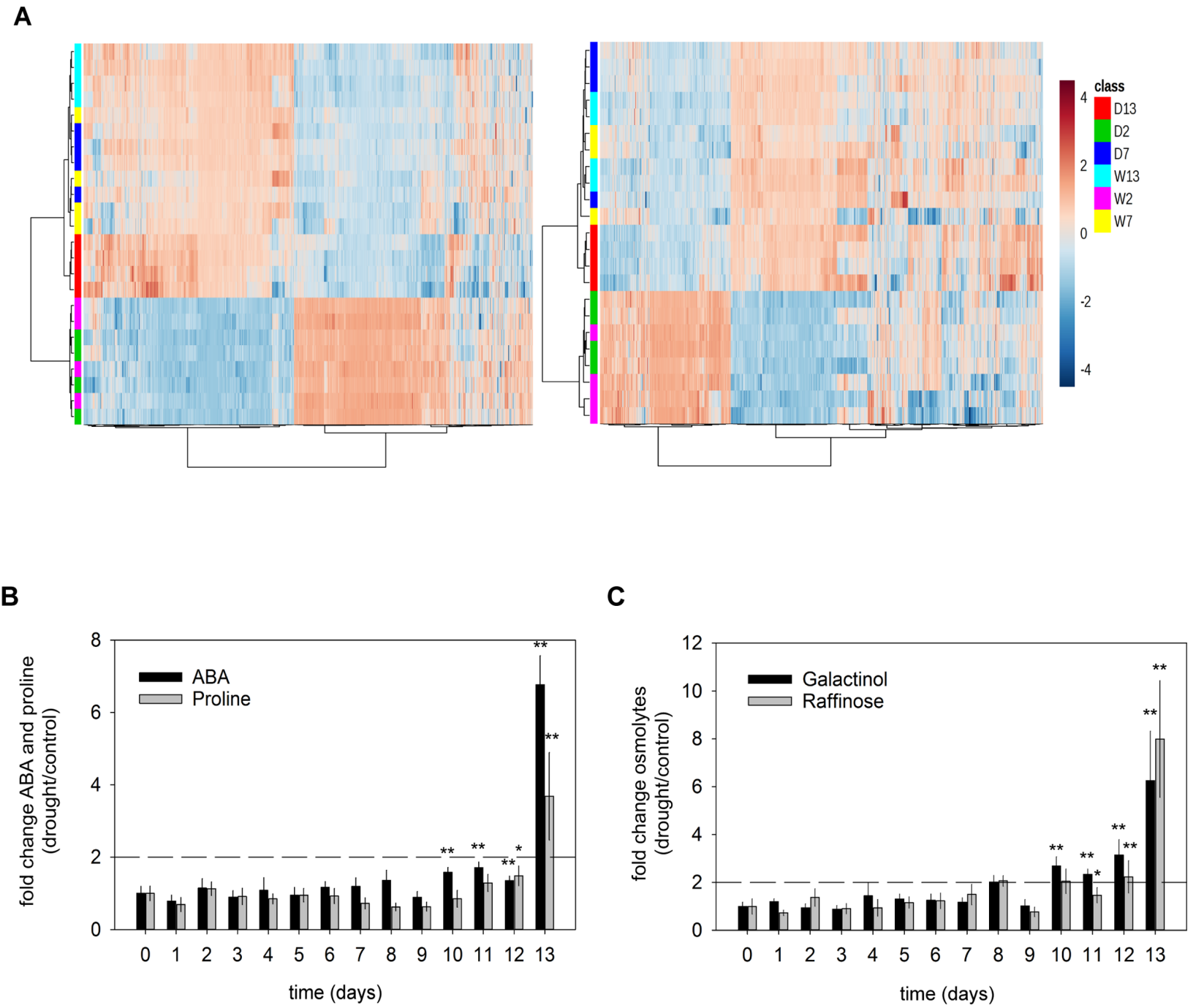

Figure 3: Metabolite levels during progressive drought. A - LC-ESI-QToF MS metabolite profiling of Arabidopsis leaves under well watered (W) and progressive soil drought (D) conditions. Leaf extracts were analysed in negative and positive ionisation modes. The heat maps show the normalised abundances of all detected chemical features. Samples and chemical features were clustered using a Pearson distance measure and the Ward clustering algorithm (see Supplemental Data Set 18); **B – r**elative concentrations of ABA (black bars) and proline (grey bars), and **C** - relative concentrations of galactinol (black bars) and raffinose (grey bars). The data represent the mean of the ratio (n=4; ± s.e.m; * $p < 0.05$, or ** $p < 0.01$).

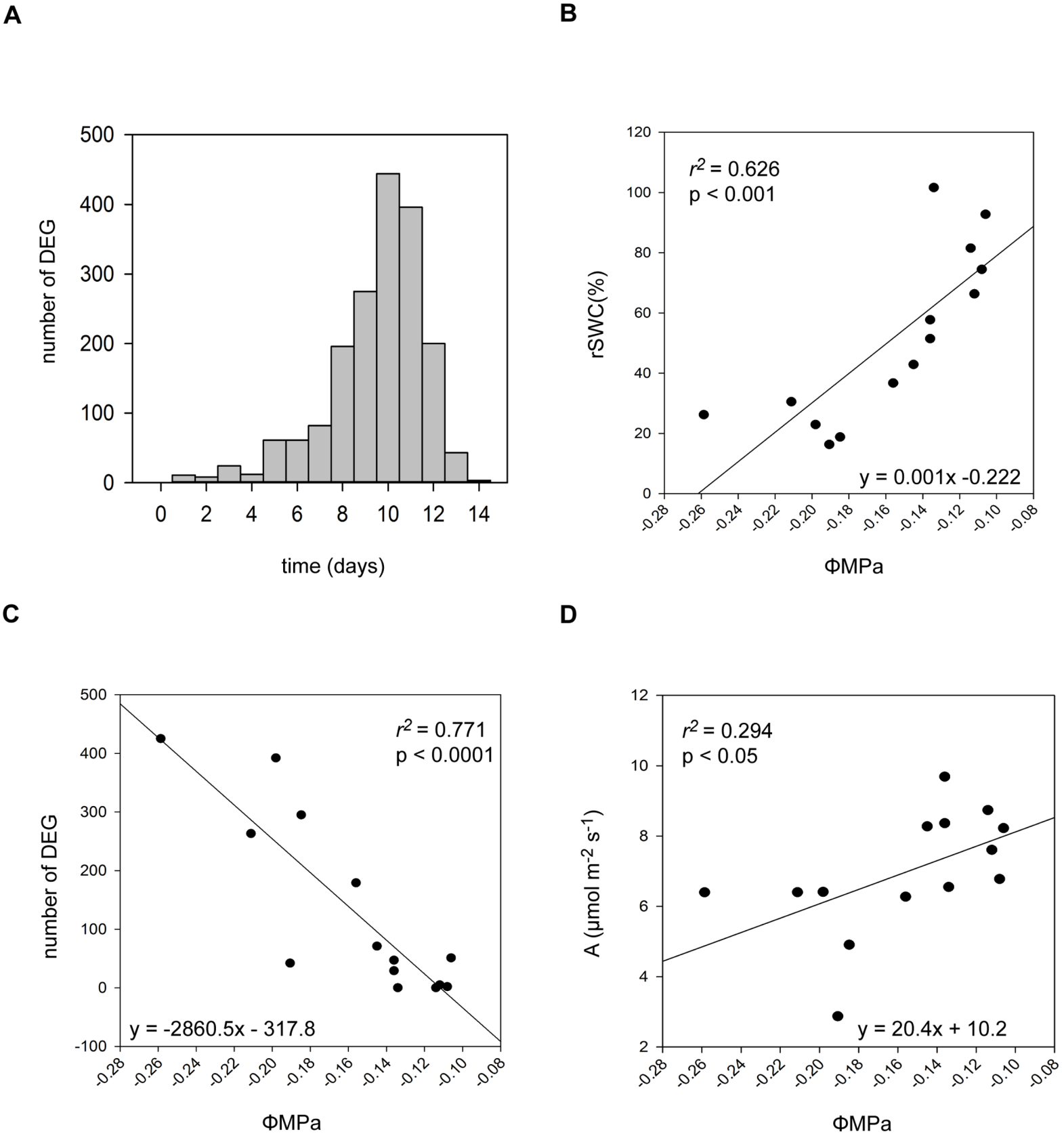

Figure 4: The relationship between stress severity and differential gene expression. **A** – number of genes for which the first differential expression was observed at each time point, indicating a late transcriptional response; **B** – correlation between leaf water potential and rSWC; **C** – correlation between leaf water potential and number of differentially expressed genes, and **D** – correlation between carbon assimilation and leaf water potential. Line represents the linear regression, r^2 and p values are given.

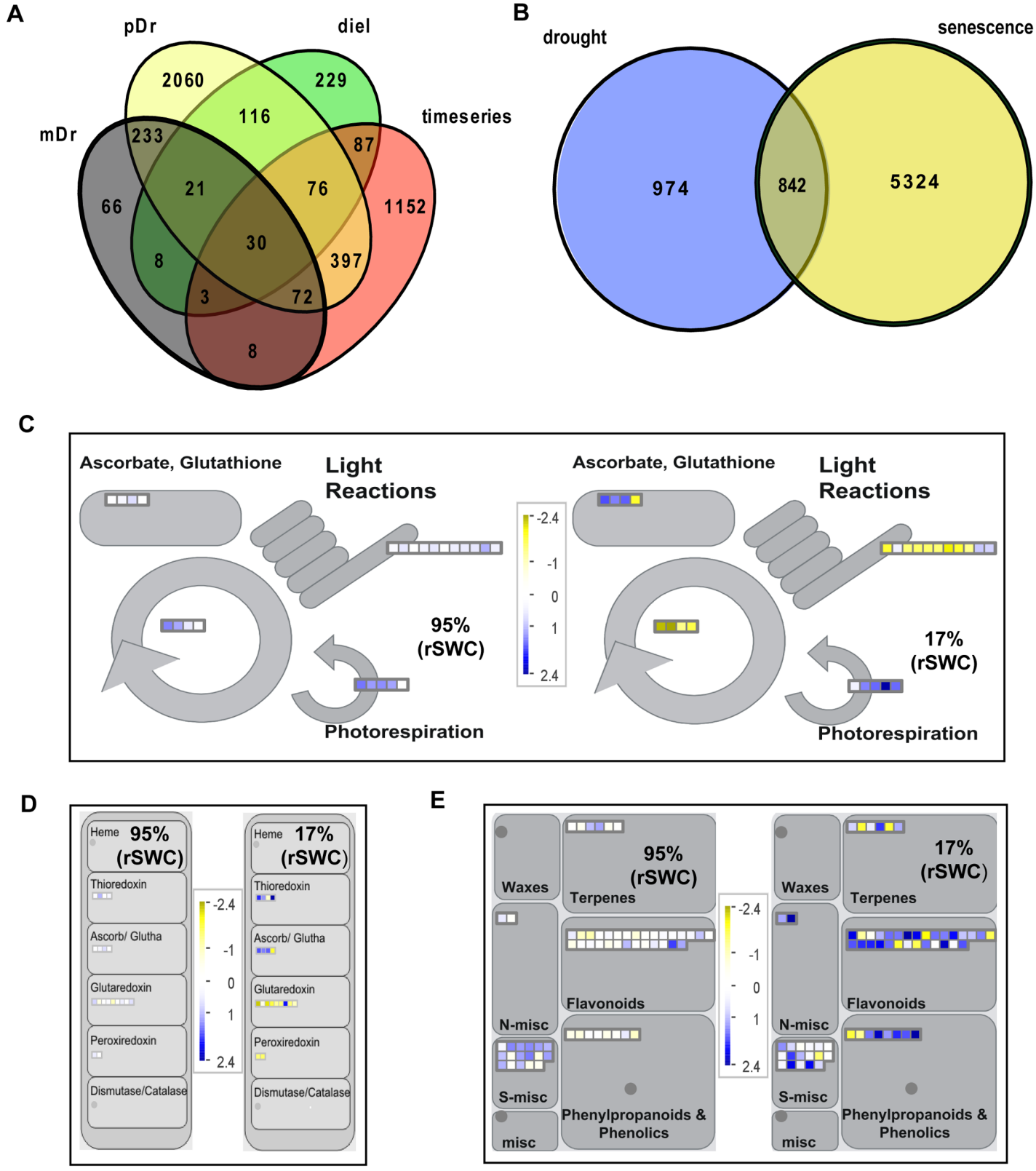

Figure 5: Comparative meta-analysis with publicly available datasets, and MapMan analysis of primary and secondary metabolism pathways. A - Comparative meta-analysis of the 1815 DEGs with publicly available drought datasets. The Venn diagram shows the overlap of time series DEGs with those responsive to moderate (mDr; Harb et al., 2010) or progressive drought (pDr; Harb et al., 2010), and a moderate drought at different times of day (diel, Wilkins et al., 2010); **B** - Comparative meta-analysis of the 1815 DEGs with a publicly available leaf 7 senescence time-series dataset (Breeze et al., 2011). The Venn diagram shows the overlap of drought - and senescence DEGs; **C** - overview of antioxidant, photosynthesis and photorespiration-related gene expression at two different time points (95% rSWC and 17% rSWC); **D** – overview of oxidation/reduction-related gene expression at two different time points (95% rSWC and 17% rSWC), and **E** – overview of secondary metabolism-related gene expression at two different time points (95% rSWC and 17% rSWC). All MapMan diagrams show gene expression data in leaf 7, where blue indicates increased and yellow indicates decreased gene expression according to the scale. Each square represents a single gene within the pathways.

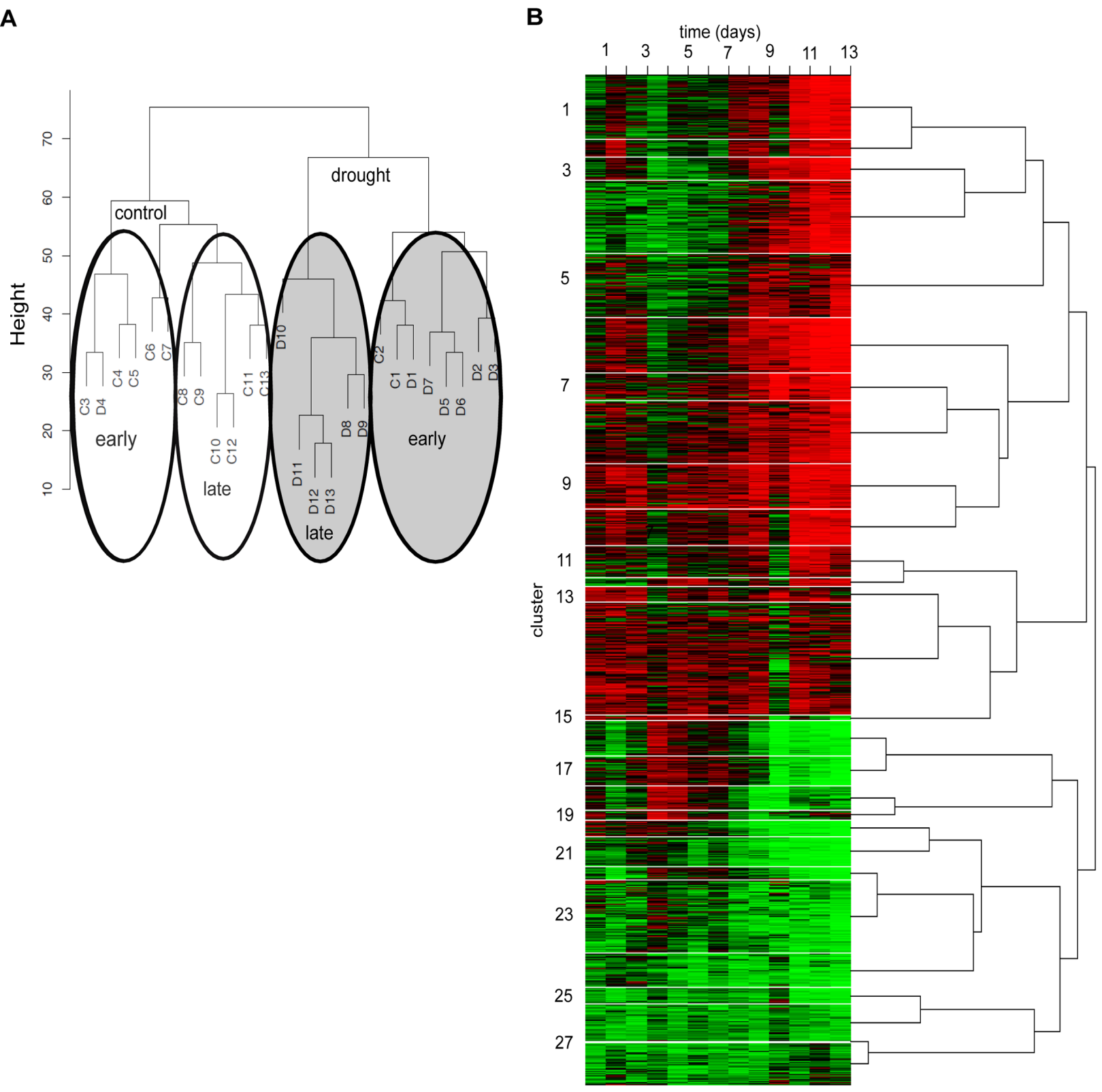

Figure 6: Temporal clustering of 1815 differentially expressed genes. A – Dendrogram of the hierarchical clustering using a Euclidian distance divides the dataset into early and late responses for both the control (white circles) and drought (grey circles) samples, and **B** - heat map of the SplineCluster analysis of the 1815 DEGs on differentially expressed genes (drought samples only) across the time series using normalised and averaged data (Supplemental Data Set 11). The heat map demonstrates expression profiles for genes in each cluster with red representing high expression and green representing low expression.

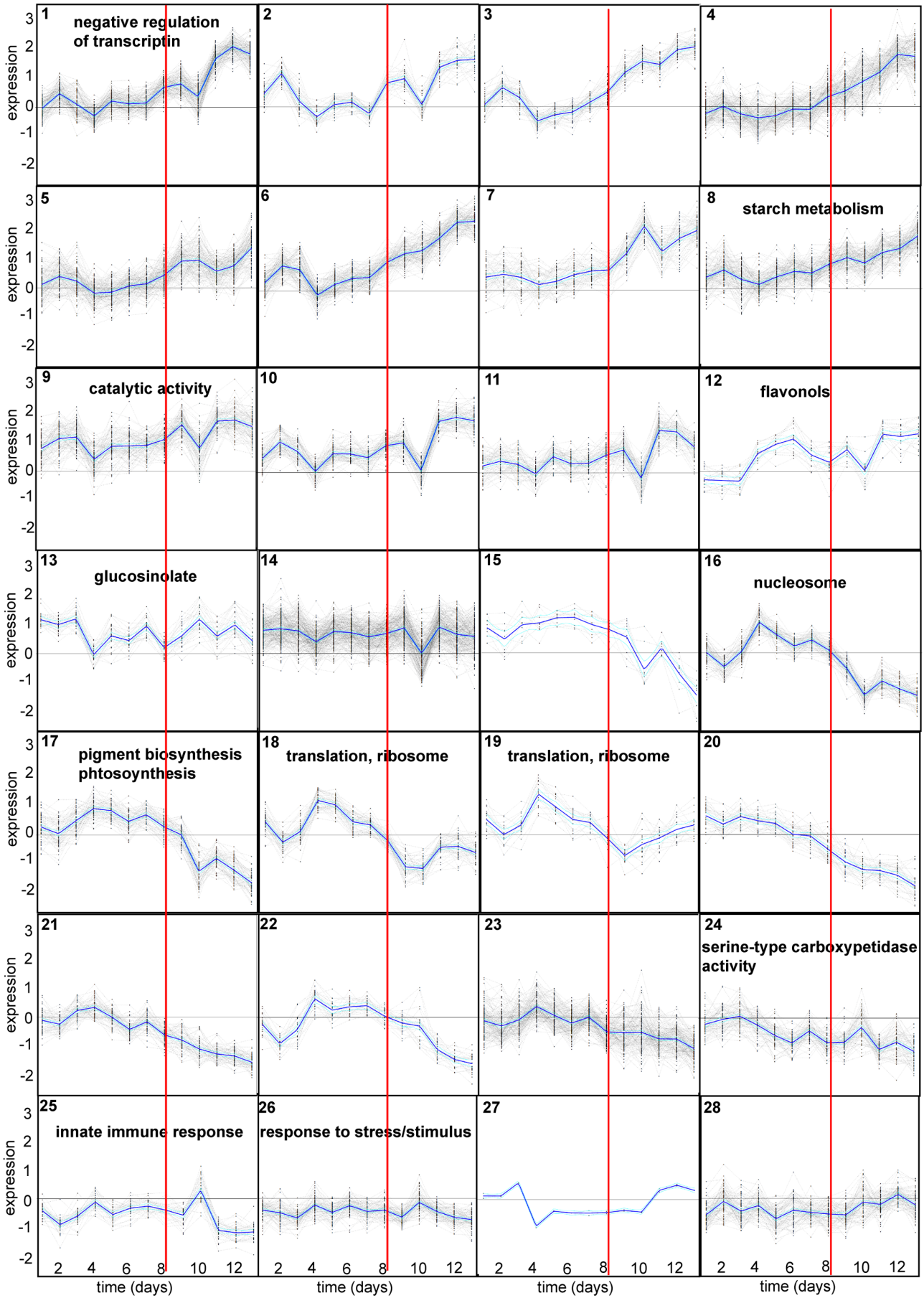

Figure 7: Descriptions of the 28 clusters derived from SplineCluster:e 7: The blue line indicates the mean expression profile for each of the 28 clusters. Individual genes present in each cluster are available in Supplemental Data Set 11. The red line indicates the switch from early (95% - 45% rSW; days 1-7) to late (40% - 17% rSWC; days 8 - 13). Selected enriched GO terms (see Supplemental Data Set 12) are indicated on each cluster.

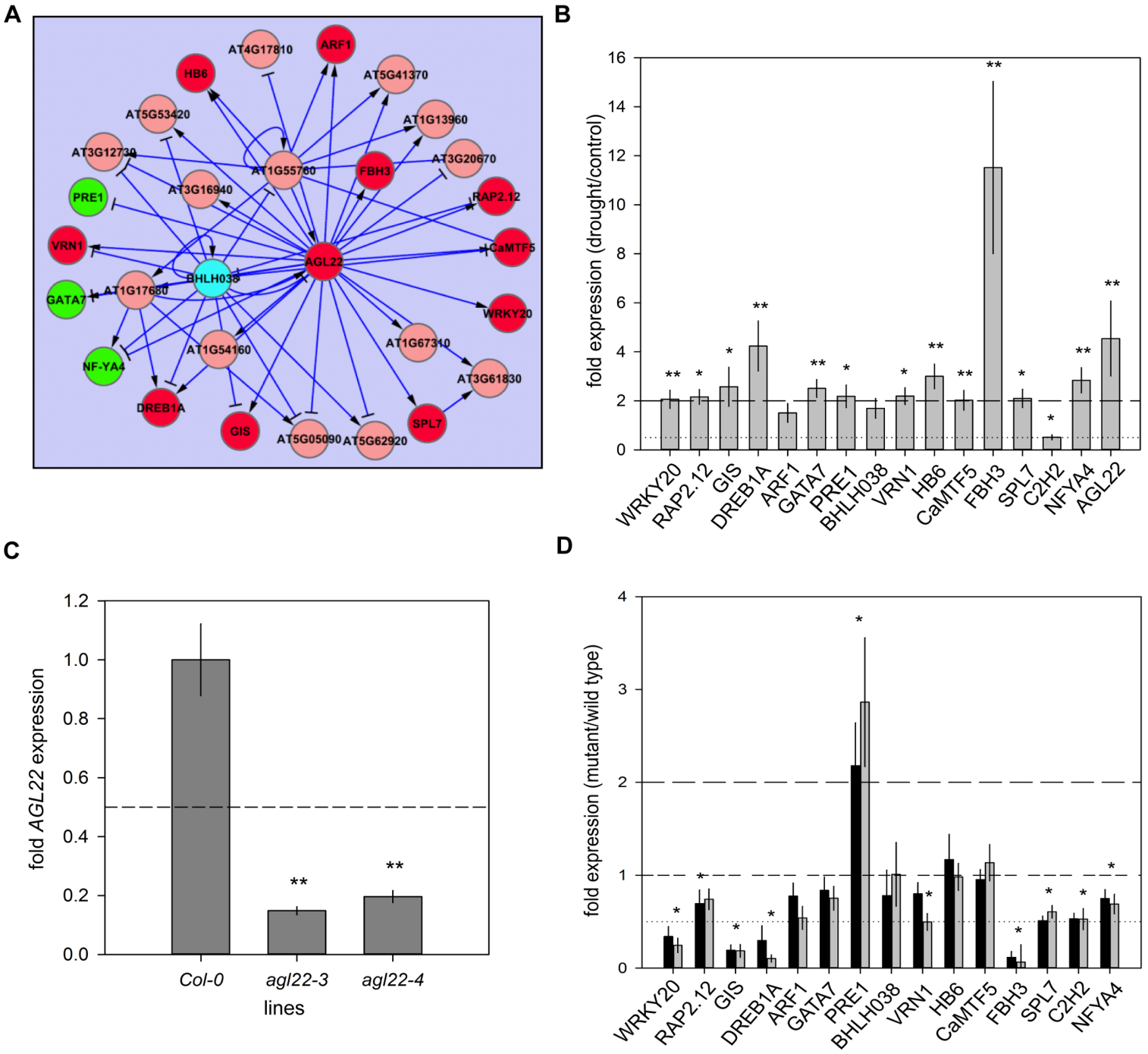

Figure 8: Constructing and evaluating a transcription factor regulatory network. A - gene regulatory network generated using VBSSM with the drought time-series data (threshold z-score = 1.65). The nodes highlighted in red were up-regulated during drought stress including the central hub gene, *AGL22*. Nodes highlighted in green were genes down regulated during drought stress. Blue nodes signify genes that were not regulated by *AGL22* as predicted from the model (see panel D). All red, green and blue nodes were selected for evaluation after drought stress; **B** - relative gene expression of selected genes under drought conditions (17% rSWC). Gene expression was analysed by qPCR. The numbers are expressed as fold changes of drought over control (n=5 ± s.e.m). Significance of the fold changes are indicated by either * $p < 0.05$, or ** $p < 0.01$. For gene and primer list see Supplemental Data Set 16 and Supplemental Table 1; **C** - relative expression levels of *AGL22* in two knockout lines, *agl22-3* and *agl22-4*, compared to wild-type determined by qPCR. Significance of the fold changes are indicated by ** $p < 0.01$, and **D** – relative gene expression profiles of 16 genes predicted to be regulated by *AGL22* under drought stress in *agl22-3* (black bars) and *agl22-4* (grey bars) compared to wild type. The data represent the mean (n =7; ± s.e.m.), significance of the fold changes are indicated by either * $p < 0.05$, or ** $p < 0.01$. Stars located centrally indicate both mutants are significantly different to wild type, while stars located over one mutant indicate significance for the specific mutant.

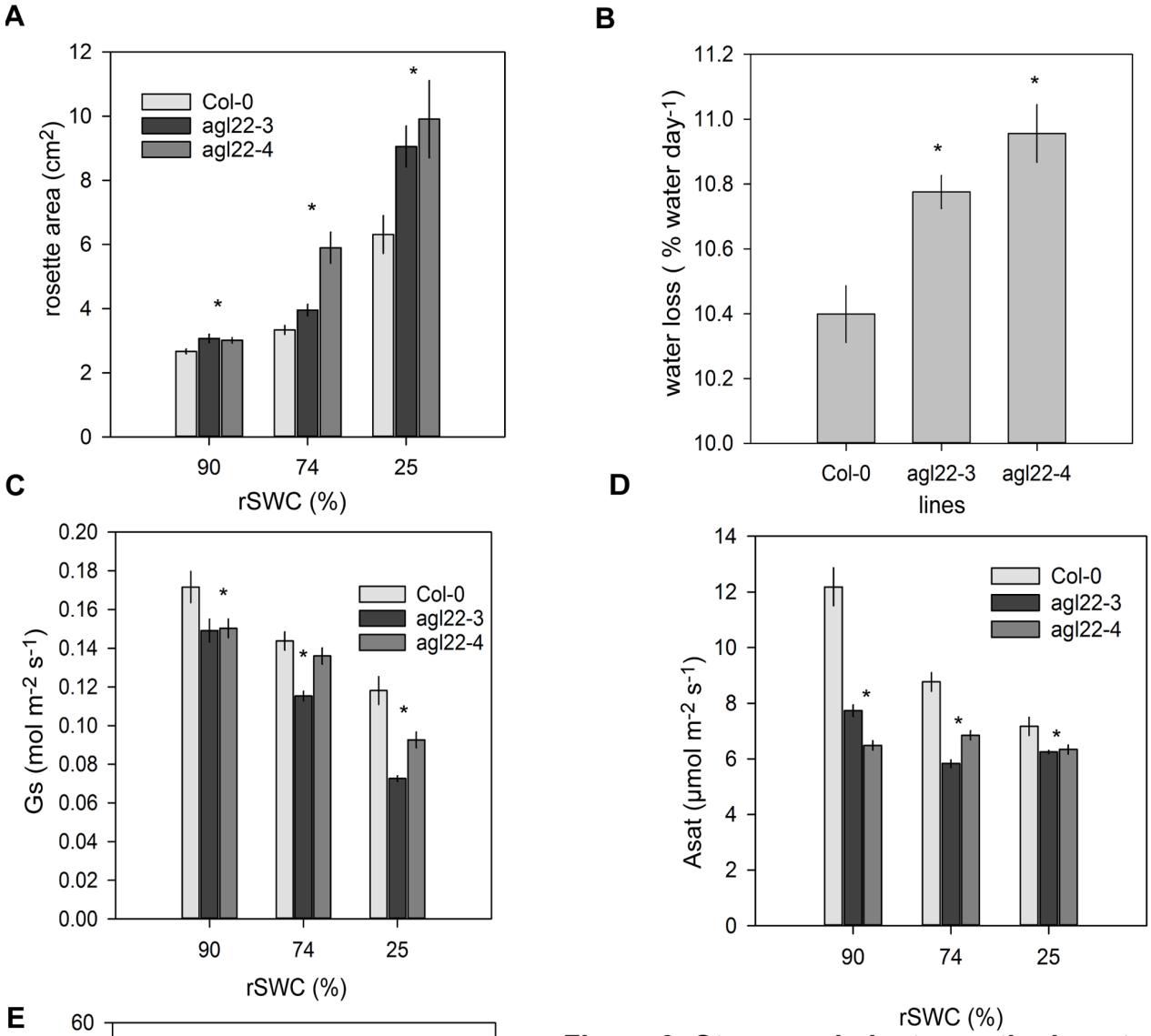

Figure 9: Stress and plant growth phenotypes of *agl22* mutants. Due to the early-flowering phenotype of both *agl22* mutant alleles, drought stress was begun at 22-days after sowing. **A** - rosette area (cm2) of Col-0 (light grey), *agl22-3* (black) and *agl22-4* (dark grey) plants at different soil water contents (n=5). The * indicates significant difference compared to wild type at $p < 0.05$; **B** - rate of water loss in *agl22-3* and *agl22-4* plants compared to wild type averaged over 13 days water withdrawal (n = 10). The * indicates significant difference compared to wild-type at $p < 0.05$; **C** - stomatal conductance (Gs) at different soil water contents in Co-0 (light grey), *agl22-3* (black) and *agl22-4* (dark grey), (n=5). The * indicates significant difference compared to wild-type at $p < 0.05$; **D** – light saturated carbon assimilation (Asat) at different soil water contents in Col-0 (light grey), *agl22-3* (black) and *agl22-4* (dark grey; n=5). The * indicates significant difference compared to wild type at $p < 0.05$, and **E** - days to flowering in well-watered (light grey) and drought-stressed (black) plants (n=10). Plants were grown under short day conditions as described in the Methods section. At 5 weeks, plants were subjected to progressive drought stress. When 17% rSWC was reached, plants were re-watered and flowering time was recorded as days after sowing. Control plants were maintained well watered. The * indicates significant difference compared to wild type at $p < 0.05$.

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