Running title: ATAF1 regulates TREHALASE1

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Research Area: Biochemistry and Metabolism
Transcription factor ATAF1 integrates carbon starvation responses with trehalose metabolism

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Summary:
The transcription factor ATAF1 from \textit{Arabidopsis thaliana} regulates \textit{TREHALASE1} expression and induces a carbon starvation transcriptome and metabolome.

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Plants respond to low carbon supply by massive reprogramming of the transcriptome and metabolome. We demonstrate here that the carbon starvation-induced NAC transcription factor *ATAF1* from *Arabidopsis thaliana* plays an important role in this physiological process. We identified *TREHALASE1 (TRE1)*, the only trehalase-encoding gene in Arabidopsis, as a direct downstream target of ATAF1. Overexpression of ATAF1 activates *TRE1* expression and leads to reduced trehalose-6-phosphate levels and a sugar starvation metabolome. In accordance with changes in expression of starch biosynthesis and breakdown related genes, starch levels are generally reduced in *ATAF1* overexpressors, but elevated in *ataf1* knockout plants. At the global transcriptome level, genes affected by ATAF1 are broadly associated with energy and carbon starvation responses. Furthermore, transcriptional responses triggered by ATAF1 largely overlap with expression patterns observed in plants starved for carbon or energy supply. Collectively, our data highlight the existence of a positively acting feed-forward loop between *ATAF1* expression, which is induced by carbon starvation, and the depletion of cellular carbon/energy pools that is triggered by the transcriptional regulation of downstream gene regulatory networks by ATAF1.
INTRODUCTION

Trehalose is a non-reducing disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units and it is found in a wide range of organisms (Avonce et al., 2006). Trehalose biosynthesis involves trehalose-6-phosphate (Tre6P) synthases (TPSs) and Tre6P phosphatases (TPPs), which catalyze the consecutive generation of Tre6P from glucose-6-phosphate and UDP-glucose, and the dephosphorylation of Tre6P to trehalose. The Arabidopsis thaliana genome contains 11 TPS genes of which TPS1 (Blazquez et al., 1998) as well as TPS2 and TPS4 (Delorge et al., 2015) encode catalytically active enzymes, while all 10 TPP genes encode active enzymes conferring spatiotemporal control over trehalose metabolism (Vandesteene et al., 2012). Trehalose is catabolized into two glucose monomers by trehalase, a plasma-membrane-associated enzyme with its catalytic domain oriented towards the apoplast (Frison et al., 2007). In Arabidopsis, trehalase is encoded by a single gene (TREHALASE1, TRE1) (Leyman et al., 2001; Müller et al., 2001; Lunn, 2007). The level of Tre6P reflects sucrose concentration and has been widely accepted as a signal for sucrose status in plants (Lunn et al., 2006; Lunn et al., 2014). Tre6P is necessary for carbon utilization and growth, although the molecular mechanisms through which it regulates plant growth are not known in detail (Schluepmann et al., 2003; Zhang et al., 2009; Schluepmann et al., 2012; Martins et al., 2013; Yadav et al., 2014).

Snf1-related kinase 1 (SnRK1) is the plant homolog of the yeast (Saccharomyces cerevisiae) Snf1 protein kinase and animal AMP-activated protein kinases, and it consists of the three subunits α, β and γ (Halford and Hey, 2009). In Arabidopsis, the catalytic α-subunit is encoded by two homologous genes, i.e., KIN10 and KIN11. Transient overexpression of KIN10 in protoplasts causes massive transcriptional reprogramming which resembles expressional patterns induced by sugar starvation, indicating that SnRK1 plays a role in mediating the energy deficiency response by repressing anabolic processes that utilize carbon to promote growth (Baena-Gonzalez et al., 2007). In addition, accumulation of Tre6P in response to high sucrose levels has been proposed to inhibit SnRK1 activity in developing tissues (Zhang et al., 2009; Paul et al., 2010); whether this inhibition operates in a simple linear manner or as part of a more complex regulatory network is a matter of debate (Lunn et al., 2014). Of note, transcription factors (TFs) including the NAC family member ATAF1 and the ABI3VP1 family protein FUSCA3 interact with SnRK1 (Kleinow et al., 2009; Tsai and Gazzarrini, 2012), however, the physiological role of these interactions is unknown at present. ATAF1 has been reported to negatively regulate the expression of stress-responsive genes in drought-stressed plants and upon treatment with the phytohormone ABA, and an involvement
in the physiological response to drought stress has been described (Lu et al., 2007; Jensen et al., 2008; Wu et al., 2009). In one study, overexpression of ATAF1 in transgenic Arabidopsis plants led to enhanced drought tolerance (Wu et al., 2009), while earlier studies reported a superior drought tolerance in \textit{ataf1} knock-out mutants (Lu et al., 2007; Jensen et al., 2008), suggesting that the response to drought might be affected by additional physiological parameters. As shown by Jensen et al. (2013), ATAF1 is a direct upstream transcriptional regulator of NCED3, which encodes a key enzyme of ABA biosynthesis. In accordance with this, overexpression of ATAF1 enhances \textit{NCED3} expression and triggers an increase in ABA level, while \textit{NCED3} transcript abundance was found to be lowered in \textit{ataf1} knock-out mutants compared to wild type. The increased drought tolerance observed by Wu et al. (2009) for \textit{ATAF1} overexpressors is in line with a regulatory effect of ATAF1 on \textit{NCED3}. Other known direct targets of ATAF1 are the \textit{ORE1} and \textit{GLK1} transcription factors (Garapati et al., 2015). \textit{ORE1} (also called \textit{ANAC092}) encodes a key positive regulator (a NAC transcription factor) of senescence (Kim et al., 2009; Balazadeh et al., 2010), while GLK1 is an important transcriptional regulator (besides GLK2) of chloroplast development and maintenance (Waters et al., 2008; Waters et al., 2009). Notably, while ATAF1 positively affects the expression of \textit{ORE1}, it negatively regulates the expression of \textit{GLK1}, thereby promoting senescence (Garapati et al., 2015). In accordance with its function as a positive regulator of senescence, \textit{ATAF1} expression increases during developmental and salinity stress-induced leaf senescence (Balazadeh et al., 2008; Allu et al., 2014).

Here, we demonstrate that ATAF1 directly regulates the expression of \textit{TRE1} and induces global transcriptomic changes that largely overlap with expression profiles observed in carbon- or energy-starved plants, and are similar to the SnRK1-induced carbon starvation response. Our results thus provide a molecular link between physiological processes triggered by energy deficiency and downstream transcriptional output to adjust plant growth and physiology.

**RESULTS AND DISCUSSION**

**ATAF1 Regulates Trehalose Metabolism**
To identify target genes of ATAF1 we generated transgenic Arabidopsis plants expressing ATAF1 under the control of a chemically (estradiol, ESTR) inducible promoter (ATAF1-IOE lines) and performed transcriptome profiling using Affymetrix ATH1 micro-arrays on samples harvested 1, 2, and 5 hours after ESTR induction (for experimental details see Methods). Several genes encoding enzymes of trehalose metabolism were found to be transcriptionally affected. To confirm this observation, we re-assessed the expression of trehalose metabolism-related genes by qRT-PCR in 2-week-old ATAF1-IOE seedlings after 5 h of ESTR treatment, as well as in 35S:ATAF1 overexpressors and a T-DNA insertion line (homozygous ataf1-4 knockout mutant from the GABI-Kat collection, GK565H08). As seen in Figure 1A and Supplemental Table I, expression of TPS1, TPS5, TPP-A, TPP-F, and TPP-H was reduced, while expression of TPS8, TPS10, TPS11, TPP-C, TPP-E, and TRE1 was elevated in ESTR-treated ATAF1-IOE seedlings and 35S:ATAF1 plants. The opposite was observed in ataf1-4, indicating that ATAF1 plays a role in transcriptional regulation of the synthesis and breakdown of intermediates of trehalose metabolism. We therefore determined the levels of Tre6P and its substrates in seedlings of wild-type (WT) and ATAF1-modified plants. The level of Tre6P was significantly reduced in 35S:ATAF1, but increased in ataf1-4 compared to WT (Figure 2A). The levels of uridine-diphosphoglucose (UDPG) and glucose-6-phosphate (G6P), the substrates...
for Tre6P synthesis, also had a contrasting pattern in 35S:ATAF1 and ataf1-4 plants compared to WT (Figure 2B and 2C).

TRE1 is a Direct ATAF1 Target

Overexpression of ATAF1 led to increased TRE1 expression (Figure 1A) and trehalase protein level (Figure 1B). Analysis of the promoters of trehalose metabolism-related genes revealed the presence of an ATAF1 binding site (VDHVNNYRR(N6)YACGNMWSK) in TREHALASE1 (TRE1) at position -561 to -538 bp upstream of the transcription start site suggesting it to be a direct downstream target of ATAF1. To test this possibility, we performed an electrophoretic mobility shift assay (EMSA) and observed binding of ATAF1 to a double-stranded DNA probe containing the ATAF1 binding site of the TRE1 promoter (Figure 1C). To provide further evidence of in vivo regulation of TRE1 by ATAF1, we performed chromatin-immunoprecipitation (ChIP) using a transgenic line that expresses the ATAF1-GFP fusion protein under control of the cauliflower mosaic virus (CaMV) 35S promoter. Elevated TRE1 expression was observed in the 35S:ATAF1-GFP line (Figure 1D), confirming that the fusion protein was functional, and an enrichment of TRE1 promoter
fragments in the precipitated chromatin was determined by quantitative PCR (qPCR) using primers spanning the ATAF1 binding site (Figure 1E).

Growth of ATAF1-modified Plants on Trehalose Medium

Trehalose leads to toxic effects when present at high concentration in the medium (Schlöpflm et al., 2004). In Arabidopsis, seedlings fail to develop rosette leaves and exhibit stunted root growth when grown on medium supplemented with trehalose; this effect is even more pronounced in tre1 knockout mutants, while TRE1 overexpressors show improved growth, likely due to the capacity of the apoplastic trehalase to catabolize external trehalose thereby avoiding toxicity (Van Houtte et al., 2013). As expression of TRE1 was reduced in ataf1-4 but enhanced in 35S:ATAF1 plants, we tested their growth on trehalose-containing medium, revealing reduced root elongation in ataf1-4 seedlings, but increased elongation in 35S:ATAF1 seedlings compared to WT (Figure 3A and 3B). Notably, the enhanced root growth of 35S:ATAF1 plants was suppressed on trehalose medium supplemented with validamycin A, an inhibitor of trehalase, suggesting that increased root elongation was triggered by enhanced trehalase activity compared to controls, which we indeed observed in ESTR-induced (10 h) ATAF1-IOE and 35S:ATAF1 seedlings (Figure 3C and 3D). Concomitant with elevated trehalase activity in these lines, trehalose levels were reduced in 35S:ATAF1 seedlings compared to WT grown on trehalose medium (Figure 3E).

Shoots of trehalose-grown seedlings accumulate starch likely due to: (i) transcriptional induction of APL3 that encodes one of four isoforms of the large subunit of ADP-glucose pyrophosphorylase (AGPase), a key enzyme of starch biosynthesis (Neuhaus and Stitt, 1990; Vigeolas et al., 2004), and (ii) an impaired export of sucrose to sinks (Wingler et al., 2000). Furthermore, inhibition of root growth on trehalose correlates with impaired expression of genes involved in starch breakdown such as STARCH EXCESS1 (SEXI) and BETA-AMYLASE3 (BAM3), and of the SUGAR TRANSPORTER1 (STP1) gene, resulting in altered sugar allocation to, or perception in the root (Ramon et al., 2007).

To test a possible function of ATAF1 in this process, we measured the expression of starch synthesis (APL3, GBSS1, SS3, BE1, ISA2/DBE1), starch breakdown (GWD3, BAM1, BAM3, BAM4) and sugar transport (SWEET4, SWEET7, SWEET11, SWEET12, SWEET15, STP1) -related genes in ATAF1 plants grown on trehalose medium. As shown in Figure 3F, genes encoding enzymes of starch biosynthesis showed elevated expression, while genes linked to starch breakdown and sugar transport were decreased in ataf1-4 shoots compared to WT, and opposite patterns occurred in 35S:ATAF1. The observed expression profiles suggested altered
starch turnover. Indeed, starch content in ataf1-4 seedlings grown on trehalose medium for 10-20 days was higher than in WT, and reduced in 35S:ATAF1 seedlings (Figure 3G; starch determined at midday). We next determined starch in rosette leaves of plants grown in soil with a 12-h photoperiod. At the end of the day (ED), starch levels were lower in 35S:ATAF1 than WT, while there was no difference between ataf1-4 and WT. However, at the end of the night (EN), ataf1-4 plants retained slightly more starch than WT, likely due to ATAF1-induced changes in starch metabolism (Figure 3H and Supplemental Figure 1), in accordance with the expression of native ATAF1 which is higher at EN than ED in WT.

The above reported changes in starch accumulation in ATAF1-modified plants might not only be due to transcriptional control exerted by ATAF1 over starch biosynthesis and breakdown...
related genes, but might also involve Tre6P itself as a regulatory molecule, which has been proposed to negatively affect starch breakdown, although the biochemical mechanism through which this control is exerted remains unknown at present (Martins et al., 2013).

It was previously reported that overexpression of KIN10 or the transcription factor bZIP11 confers trehalose resistance without a significant induction of trehalase activity (Delatte et al., 2011), which differs from the situation shown here for ATAF1 overexpressors exhibiting elevated trehalase activity. Together these observations suggest that trehalase contributes to, but may not be the only factor supporting tolerance to exogenously supplied trehalose.

**ATAF1 Induces a Carbon Starvation Transcriptome**

It was shown earlier that transient overexpression of KIN10 (encoding the α-subunit of SnRK1) in protoplasts causes transcriptional reprogramming with 506 up- and 515 downregulated genes (2-fold cut-off); the expression pattern resembled that of sugar starved cells (Baena-Gonzalez et al., 2007). As ATAF1 overexpressors had decreased levels of Tre6P, possibly signaling low-sucrose availability (Lunn et al., 2006; Lunn et al., 2014), we compared the transcriptome data of ESTR-treated ATAF1-IOE seedlings and mature leaves with the 1,021 genes modified by KIN10 overexpression. As shown in Supplemental Figure 2, the number of KIN10-affected genes increased over time in ATAF1-IOE samples (see also Supplemental Table II). Thus, the ATAF1-IOE transcriptome became progressively more similar to the SnRK1-induced expression profile, which resembles carbon starvation-induced transcript profiles (Baena-Gonzalez et al., 2007).

Further, we tested the expression of a subset of randomly chosen genes that are upregulated both by ATAF1 and KIN10 overexpression in seedlings transferred onto trehalose medium. As shown in Figure 4A, the expression of SnRK1-upregulated genes was elevated in 35S:ATAF1 seedlings, while their expression was reduced in ataf1-4 compared to WT, demonstrating an upregulation of SnRK1-mediated transcriptional responses upon ATAF1 overexpression. Thus, 35S:ATAF1 seedlings appear to adopt a transcriptional status similar to that of protoplasts transiently overexpressing SnRK1.

Previously, global transcriptional responses to changes of endogenous sugar availability were identified by comparing Arabidopsis plants illuminated for 4 h at ambient or low (< 50 ppm) CO₂ concentration (Bläsing et al., 2005). As ATAF1 was among the genes strongly repressed at ambient CO₂ (Supplemental Table 6M of Bläsing et al., 2005; Supplemental Figure 3), we compared the ATAF1-induced transcriptional responses with the top 200 genes induced or repressed upon carbon fixation (Bläsing et al., 2005). As seen in Figure 5A and 5B (see also...
Supplemental Table III), genes induced by carbon fixation were repressed in ATAF1-IOE, and vice versa.

We next compared the ATAF1-controlled genes with: (i) genes affected by addition of sucrose (15 mM, 30 min and 3 h) or glucose (100 mM, 3 h) to carbon-starved seedlings (Bläsing et al., 2005; Osuna et al., 2007); and (ii) genes that are altered at the end of the day (carbon rich) compared to the end of the night (carbon poor) in Col-0 wild type and starch-deficient phosphoglucomutase (pgm) mutant plants (Bläsing et al., 2005). Notably, genes repressed by carbon re-supply / high-energy status of the plant are induced in ATAF1-IOE overexpressors (Figure 6A), while conversely genes induced by carbon re-addition / high energy status are repressed (Figure 6B; see also Supplemental Table IV). Furthermore, genes induced or repressed by ATAF1 overexpression, respectively, largely overlap with genes induced or repressed under conditions of extended night (Usadel et al., 2008); Figure 6C and Supplemental Table V). Thus, the transcriptome data clearly indicate that ATAF1 induces a transcriptional reprogramming that is highly similar to gene expression changes observed under conditions of carbon or energy limitation.
To gain a wider insight into the cellular processes affected by ATAF1, we clustered the genes affected by its expression within 1 - 5 h of estradiol treatment (in ATAF1-IOE lines) using the Short Time-series Expression Miner (STEM; Ernst and Bar-Joseph, 2006). STEM analysis identified three significant clusters with genes upregulated over time (profiles 13, 8 and 12) and three clusters with genes downregulated (profiles 7, 2, and 3) after ATAF1 induction (Supplemental Figure 4A; Supplemental Table VI). Analysis of the GO terms significantly associated with genes in the upregulated profiles include ‘response to water deprivation’, ‘autophagy’, ‘galactolipid metabolic process’ and ‘cellular response to phosphate starvation’ (Supplemental Figure 4B), while those associated with genes downregulated by ATAF1 include ‘carbohydrate biosynthetic process’, ‘sulfur compound biosynthetic process’, ‘pentose-phosphate shunt’ and ‘starch biosynthetic process’ (Supplemental Figure 4C). Thus, the observed transcriptome responses further support the notion that ATAF1 induces a physiological response reminiscent of carbon or energy deprivation.

Autophagy and Amino Acid Catabolism Genes are Upregulated by ATAF1
Autophagy is a cellular process that results in the degradation of cytoplasmic components upon carbon starvation (Contejo et al., 2004; Avin-Wittenberg et al., 2015), concomitant with the induction of autophagy-related genes such as ATG3, ATG4a, ATG4b, ATG7 and ATG8a - ATG8i in Arabidopsis (Rose et al., 2006). Likewise, genes encoding enzymes involved in amino acid breakdown such as isovaleryl-CoA dehydrogenase (IVDH), D-2-hydroxyglutarate dehydrogenase (D2HGDH) and glutamate dehydrogenase (GDH), and genes encoding mitochondrial electron-transfer flavoprotein/electron transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETF-QO) subunits are upregulated upon carbon limitation (Ishizaki et al., 2006; Miyashita and Good, 2008; Araujo et al., 2011; Izumi et al., 2013). IVDH and D2HGDH act as electron donors to the ETF/ETF-QO-mediated alternative pathway of respiration (Araujo et al., 2010). Thus, during sugar starvation, autophagy contributes to amino acid catabolism, thereby allowing use of free amino acids as alternative respiratory substrates.

To confirm the molecular carbon starvation phenotype of ATAF1 transgenic lines, we used qRT-PCR to test the expression of genes of all three metabolic pathways and observed strong induction in ATAF1-IOE and 35S:ATAF1 plants, while expression of the tested genes was not significantly different between ataf1-4 and WT (Figure 4B and Supplemental Table I).
However, expression of these genes was reduced in dark-incubated leaves of soil-grown ataf1-4 mutants compared to WT (Figure 4B), suggesting that loss of ATAF1 results in a delayed launch of autophagy, which is in accordance with the delayed dark-induced senescence of ataf1 knockout mutants (including ataf1-4), as previously reported (Garapati et al., 2015).

ATAF1 Triggers Sucrose Starvation-type Changes in Primary Metabolism

Previous research demonstrated that sugars and glycolytic intermediates are depleted in carbon-starved seedlings, while their levels increase after sucrose resupply (Lunn et al., 2006). It was also shown that Tre6P controls primary metabolism and is essential for carbohydrate utilization and plant growth (Schluepmann et al., 2003). As ATAF1-modified plants showed altered levels of Tre6P, we investigated whether perturbed expression of ATAF1 has an influence on central sugar metabolism, by measuring primary metabolites in seedlings of WT and ATAF1-modified plants (Table I). The levels of glycolytic intermediates were higher in the ataf1-4 mutant and lower in 35S:ATAF1 plants when compared to WT. Additionally, the levels of soluble sugars (glucose, fructose and sucrose) were lower and higher, respectively, in 35S:ATAF1 and ataf1-4 plants (Figure 4C). Thus, the metabolic changes in 35S:ATAF1 plants mimic those occurring during sucrose starvation (Lunn et al., 2006).

Model of ATAF1 Action

Based on the data presented here and elsewhere (Bläsing et al., 2005; Baena-Gonzalez et al., 2007; Zhang et al., 2009; Paul et al., 2010; Lunn et al., 2014; Yadav et al., 2014), we propose a model for the regulatory integration of ATAF1 (Figure 7): Expression of ATAF1 is strongly induced by carbon limitation triggered by environmental input, e.g. an extended night (Usadel et al., 2008), dark incubation of whole plants (Lin and Wu, 2004; Garapati et al., 2015), drought stress (Lu et al., 2007; Wu et al., 2009), or low atmospheric CO2 concentration (Bläsing et al., 2005), leading directly and indirectly to reprogramming of the transcriptome and metabolome. This response includes the direct transcriptional activation of the TRE1 gene by ATAF1. In addition, ATAF1 may act through transcriptional regulation of other carbon metabolism-related genes (cf. Figure 3F). Enhanced expression of ATAF1 triggers a reduction of cellular trehalose, Tre6P, sucrose and starch levels. Low-carbon status may furthermore act through currently unknown mechanisms by which low carbon (potentially sucrose) inhibits TPS1 or activates TPP, respectively, as suggested previously (Yadav et al.,...
ATAF1 might also lead to transcriptional reprogramming by reducing cellular Tre6P level and a concomitant activation of SnRK1 protein kinase activity (indicated as “SnRK1 regulation” in Figure 7), although it currently remains unknown how exactly Tre6P and SnRK1 activity are related in vivo and how SnRK1 controls the expression of genes affected by ATAF1.

**Figure 7: Model of ATAF1 Action.** Reduced carbon availability triggered by environmental input (e.g., extended darkness, low atmospheric CO₂ concentration, drought) induces ATAF1 expression, while high expression of ATAF1 itself causes a low-carbon physiological status, suggesting the existence of a carbon starvation feed-forward loop that involves ATAF1 as a regulatory node. ATAF1 directly upregulates expression of TRE1 by binding to its promoter, and additionally affects the expression of many other carbon starvation-related genes, directly or indirectly (indicated by a boxed question mark). X and Y indicate unknown mechanisms by which low carbon (potentially low sucrose) inhibits TPS and/or activates TPP, respectively (Yadav et al., 2014). Solid lines indicate direct regulatory interactions or metabolite conversions, while dashed lines indicate interactions of unknown molecular mechanism. Arrow- and T-ending lines represent activation and repression, respectively. ATAF1 may also lead to transcriptional reprogramming by virtue of the lowered Tre6P level it induces and a concomitant activation of SnRK1 activity (indicated as “SnRK1 regulation”) as suggested by other authors (Zhang et al., 2009; Nunes et al., 2013).
In summary, we have identified a positive feedback loop between ATAF1 expression and the depletion of cellular carbon pools, representing an intriguing example for the control of carbon-related physiology through the integration of transcriptional and metabolic reprogramming. Future work will have to unravel the precise involvement of other transcriptional regulators up- and downstream of ATAF1, including bZIP11, which affect trehalose metabolism genes through a currently unknown mechanism (Ma et al., 2011). Evidently, the cellular response to low carbon availability in plants is complex and ATAF1 plays an important role in the process.
METHODS

General

Unless indicated otherwise, chemicals and reagents were obtained from Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany) and Fluka (Buchs, Switzerland). Molecular biological kits were obtained from Qiagen (Hilden, Germany) and Macherey-Nagel (Düren, Germany). For sequence analyses, the tools provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), The Arabidopsis Information Resource (TAIR; http://www.Arabidopsis.org/), European Bioinformatics Institute (www.ebi.ac.uk) and the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0) were used. QuantPrime (www.quantprime.de) was used for designing qRT-PCR primers, and PatMatch (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) was used to identify ATAF1 binding sites (details will be published elsewhere) in promoters of trehalose metabolism-related genes. Sequences of oligonucleotides used for qRT-PCR, EMSA, and ChIP-PCR are given in Supplemental Table VII.

Plants

Experiments were performed using wild-type Arabidopsis thaliana (L.) Heynh., accession Col-0 (INRA, France; http://dbsgap.versailles.inra.fr/publiclines) and transgenic plants generated in this background. Seeds of the ataf1-4 knockout mutant (GK565H08) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info) and genetically characterized before use; the mutant carries a T-DNA insertion 1073 bp downstream of the transcription start site, in exon 3. For growth under a 12-h photoperiod, seeds were germinated in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) in a phytotron with 16 h (~120 μE m⁻² s⁻¹; 20°C) light / 8 h (4°C) darkness. Two weeks after sowing, seedlings were transplanted to individual 6-cm pots and transferred to equinoctial conditions (12 h of light/12 h of dark) at constant 20°C, until harvest. For plate experiments, Arabidopsis seeds were surface-sterilized using sodium hypochlorite solution (10%) and sown on nutrient agar medium (1x Murashige and Skoog, 1% sucrose, pH 5.8). The plates were stored for two days under stratification condition (16 h light, 22°C and 8 h dark, 4°C) and then transferred to long-day growth conditions (16 h light, 22°C and 8 h dark, 18°C).

Constructs
Generation of the constructs was reported in Garapati et al. (2015). In brief: 35S:ATAF1: the ATAF1 open reading frame was inserted via Pmel/PacI sites into the pGreen0229-35S plant transformation vector (Skirycz et al., 2006). 35S:ATAF1-GFP: the ATAF1 coding sequence (without stop codon) was fused to the green fluorescent protein (GFP) open reading frame in vector pK7FWG2 (Ghent University; http://gateway.psb.ugent.be/vector/show/pK7FWG2/search/index/). The vector carries the CaMV 35S promoter upstream of the ATAF1-GFP sequence. ATAF1-IOE: the ATAF1 coding sequence was inserted via XhoI/SpeI sites into the pER8 vector (Zuo et al., 2000). Agrobacterium tumefaciens strain GV3101 (pMP90) was used for Arabidopsis transformation.

Microarray Analyses
An RNeasy Plant Mini kit (Qiagen) was used to extract total RNA from 2-week-old ATAF1-IOE seedlings that were grown in liquid 1 x Murashige-Skoog medium (1% sucrose, w/v, pH 5.8) and treated with 10 µM ESTR (dissolved in 0.1% (v/v) ethanol; control treatment: 0.1% ethanol) for 1, 2 and 5 h. Alternatively, leaves of 30-day-old plants were incubated for 5 h in liquid 0.5 Murashige-Skoog medium in the presence of 10 µM ESTR (dissolved in 0.1% (v/v) ethanol; control: 0.1% ethanol) prior to RNA extraction. Three µg of RNA were processed for use in Affymetrix ATH1 microarray hybridizations. Probe preparation and hybridization were performed by ATLAS Biolabs (http://www.atlas-biolabs.com/). Raw data (CEL files) obtained from RNA hybridisation experiments were normalized with the affyPLM package from the Bioconductor software project (Gentleman et al., 2004) using GCRMA, and data were normalized with RMA (Robust Multiple array Average) (Wu et al., 2004). Transcriptional changes were determined by subtracting the normalized signal intensity of the control samples from that of ESTR-induced samples. All four ESTR induction experiments were performed as single-replicate experiments. Clustering of differentially expressed genes (ESTR- versus mock-treated) was performed using the Short Time-series Expression Miner (STEM; http://www.cs.cmu.edu/~jernst/stem) (Ernst and Bar-Joseph, 2006). GO annotation was done using the Singular Enrichment Analysis (SEA) tool from AgriGO (http://bioinfo.cau.edu.cn/agriGO) (Du et al., 2010). Expression data are available from the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE54676. qRT-PCR using SYBR Green was performed as described (Caldana et al., 2007).

Electrophoretic Mobility Shift Assay (EMSA)
For EMSA, recombinant ATAF1-CELD fusion protein (Xue, 2005) was expressed in *Escherichia coli* strain BL21 (DE3) pLysS (Agilent Technologies) as described (Dortay et al., 2011). Protein was purified using Protino Ni-IDA 150 packed columns (Macherey and Nagel). EMSA was performed as described (Wu et al., 2012) using the Odyssey Infrared EMSA kit (Li-Cor, Bad Homburg, Germany; www.licor.com). 5’-DY682-labeled DNA fragments were purchased from Eurofins MWG Operon.

**ChIP-PCR**

Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) was performed with 2-week-old Arabidopsis seedlings expressing functional GFP-tagged ATAF1 protein from the 35S promoter (*35S:ATAF1-GFP*) employing anti-GFP antibody to immunoprecipitate protein-DNA complexes as reported (Wu et al., 2012; Kaufmann et al., 2010). Col-0 plants served as negative controls. Primers used for qPCR flanked the ATAF1 binding site within the promoter region of *TRE1*. Primers annealing to promoter regions of an Arabidopsis gene (*CLAVATA1; At1g75820*) lacking an ATAF1 binding site were employed in negative-control experiments. The ChIP experiment was run in two biological replications with three technical repetitions per assay. ChIP-qPCR data were analyzed as described (Wu et al., 2012).

**Determination of Trehalase Activity**

Three replicates of at least 30 seedlings were frozen in liquid nitrogen and homogenized. The powdered tissue (100 mg) was extracted in 1 mL of ice-cold protein extraction buffer (0.1 M MES-KOH, pH 6, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% [w/v] polyvinylpyrrolidone, and 1 mM dithiothreitol) containing protease inhibitors (Complete EDTA-free; Roche). The suspension was cleared by centrifugation (18,000 × g, 4°C, 10 min). Trehalase activity was determined as described previously (Pernambuco et al., 1996) with minor modifications. Aliquots of the protein extract were incubated in the reaction buffer (62.5 mM MES-KOH, pH 7, 125 µM CaCl₂) containing 250 mM trehalose. The glucose released was quantified using the GLUCOSE (GO) Assay Kit (GAGO-20, Sigma Aldrich). Control reactions were performed by incubating the protein extracts in reaction buffer without trehalose. This allowed measurement of the glucose level in the protein extract, which was subtracted from the glucose released due to trehalase activity in samples incubated with trehalose. The protein concentrations were determined according to (Bradford, 1976) with bovine serum albumin as standard. The glucose levels were normalized against the protein concentration to determine the specific activity.
Western Blot Analysis of TRE1

Two-week-old seedlings were harvested, frozen in liquid nitrogen and homogenized. The powdered tissue (100 mg) was extracted in 1 mL of ice-cold protein extraction buffer. After centrifugation at 14,000 × g for 15 min, soluble proteins were collected and concentrations were determined using the Bradford (1976) assay. For each sample, aliquots containing 20 μg of protein were analyzed by SDS-PAGE on duplicate 12% polyacrylamide gels. One gel was stained with 0.25% Coomassie Brilliant Blue R250 as a sample loading control, and proteins from the other gel were electro-blotted onto Protran nitrocellulose membrane (Whatman, Kent, UK) for immunological analysis. The membrane was blocked for one hour in blocking buffer (5% non-fat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween-20), followed by incubation for 1 h with polyclonal rabbit anti-TRE1 antibody (1:1,000 dilution) as a primary antibody. After washing, the membrane was incubated with IRDye 800CW donkey anti-rabbit IgG secondary antibody (Li-Cor; 1: 10,000 dilution) for 60 min at room temperature with constant shaking. Signal intensities were analyzed at 800 nm using an Odyssey Infrared Imaging System (LI-COR).

Quantification of Starch and Soluble Sugars

Sucrose, glucose, and fructose were extracted with ethanol (Scheible et al., 1997) and quantified enzymatically according to (Stitt et al., 1989). Starch was determined enzymatically in the pellets obtained after ethanol extraction of soluble sugars (Hendriks et al., 2003).

Quantification of Primary Metabolites

Tre6P and phosphorylated intermediates were extracted in chloroform-methanol and measured by high-performance anion-exchange chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described in Lunn et al. (2006), using a QTrap 5500 MS-Q3 (AB Sciex) triple quadrupole mass spectrometer. Tre6P was quantified using enzymatically calibrated standards and a [2H]-Tre6P internal standard to correct for ion suppression and matrix effects (Lunn et al., 2006). Trehalose was quantified fluorimetrically as described by Carillo et al. (2013) in the same chloroform-methanol extract used for Tre6P determination.

Microscopy

Presence of ATAF1-GFP fusion protein in transgenic plants was analyzed by confocal fluorescence microscopy (Eclipse E600 microscope; Nikon).
Treatments

For ESTR induction, 2-week-old seedlings were incubated in liquid 1 x MS medium containing 1% (w/v) sucrose and 10 µM ESTR (dissolved in 0.1% (v/v) ethanol; control: 0.1% ethanol) and kept on a rotary shaker for 1, 2, 5, or 10 h until harvest. Gene expression was determined by qRT-PCR. For trehalose and validamycin A treatment, 12-day-old seedlings were transferred to solid 0.5 MS medium supplemented with trehalose (25 mM), or trehalose (25 mM) + validamycin A (10 µM), and grown for two weeks.

AGI Codes

ACTIN2 (At3g18780); ATAF1 (At1g01720); BAM3 (At4g17090); STP1 (At1g11260); TPS1 (At1g78580); TRE1 (At4g24040). Additional AGI codes are given in the Supplemental Tables.

ACKNOWLEDGMENTS

We thank Karin Koehl and her team for plant care and Sandhya Yellina for expression and purification of ATAF1 protein.
Table I. Metabolite Concentrations (nmol g\(^{-1}\) FW). Two-week-old WT, 355:ATAF1 and 
ataf1-4 seedlings grown on one-half-strength MS + 1% (w/v) sucrose medium were harvested 
at midday (of a 16 h light period) for metabolite determinations. Means ± SD. *Statistically 
significant changes (*\(p < 0.05\); **\(p < 0.01\); Student’s \(t\) test, based on three biological 
replicates).

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<thead>
<tr>
<th>Metabolite</th>
<th>WT</th>
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<td>Suc6P</td>
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<td>Fumarate</td>
<td>863 ± 24</td>
<td>1249 ± 41 **</td>
<td>922 ± 27 *</td>
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Suc6P: sucrose-6´-phosphate; ADPG: ADP-glucose; Gal6P: galactose-6-phosphate; G1,6BP: 
glucose-1,6-bisphosphate; Glc1P: glucose-1-phosphate; Gly3P: glycerol-3-phosphate; PEP: 
phosphoenolpyruvate; Fru6P: fructose-6-phosphate; Man6P: mannose-6-phosphate; F1,6BP: 
fructose-1,6-bisphosphate; 2-OG: 2-oxoglutarate; 3-PGA: 3-phosphoglyceric acid.
FIGURE LEGENDS

**Figure 1. TRE1 is a Direct Target of ATAF1. A,** Expression of trehalose metabolism-related genes after 5 h of ESTR induction in *ATAF1-IOE* (compared to mock), and in *ataf1-4* and *35S:ATAF1* plants (compared to WT). Heat maps indicate log2 fold-change expression ratios relative to controls. Means of three biological replicates each determined in two technical replicates. Full data are given in **Supplemental Table I. B,** Immunoblot analysis of TRE1 accumulation in extracts from 2-week-old *ATAF1-IOE* seedlings (treated with 10 µM ESTR for 10 h; mock: 0.1% ethanol), WT, *ataf1-4*, and *35S:ATAF1*; Coomassie Brilliant Blue stain shows Rubisco large subunit as control. **C,** EMSA confirms binding of ATAF1 to its binding site in the *TRE1* promoter. **D,** *TRE1* expression in 2-week-old *35S:ATAF1-GFP* seedlings compared to wild type. Data represent means of two biological replicates each determined in three technical replicates. The asterisk indicates a statistically significant difference to WT (*p < 0.05; Student’s *t*-test). **E,** ChIP-qPCR confirms *in vivo* binding of ATAF1 to *TRE1* promoter. Primers span the ATAF1 binding site. As negative control, primers annealing to a promoter region of a gene (*CLV1*) lacking an ATAF1 binding site were used. Means ± SD (n = 3).

**Figure 2. Levels of Tre6P, UDPG and G6P in WT, ataf1-4 and 35S:ATAF1 Seedlings.** Two-week-old wild-type (WT), *ataf1-4* and *35S:ATAF1* seedlings grown on solid one-half strength MS medium supplemented with 1% (w/v) sucrose were harvested for metabolite determinations. Data represent means ± SD (three replicates with 10 seedlings each). Asterisks indicate statistically significant differences to WT (*p < 0.05; **p < 0.01; Student’s *t*-test).

**Figure 3. Plant Growth and Starch Accumulation. A,** Seedlings grown for 2 weeks on 0.5 MS medium + 1% (w/v) sucrose (29.2 mM) were transferred to one-half strength MS medium supplemented with sucrose (29.2 mM), trehalose (25 mM), or trehalose (25 mM) + validamycin A (10 µM), and grown for two more weeks. Note the difference in root growth on trehalose medium. **B,** Root length of seedlings two weeks after transfer to trehalose (25 mM) medium (n = 6). **C,** Trehalase activity in *ATAF1-IOE* seedlings treated with ESTR (10 µM) or mock (0.1% ethanol, v/v) for 10 h. **D,** Trehalase activity in WT, *ataf1-4* mutant and *35S:ATAF1* seedlings transferred to trehalose (25 mM) medium and grown for 10 days. Note higher trehalase activity upon *ATAF1* overexpression in (C) and (D). **E,** Trehalose content in
WT and 35S:ATAF1 seedlings grown on trehalose (25 mM) medium for 10 days. F, Heat map of expression of starch metabolism- and sugar transport-related genes. Gene expression was determined in 10-day-old seedlings grown on plates at 12 h into the light period (which was 16 h in total). Means of three biological replicates each determined in two technical replicates. Full data are given in Supplemental Table I. G, Starch content in shoots of seedlings grown for 10 days on trehalose (25 mM) medium. Samples were harvested in the middle of the 16-h light period. H, Lugol staining of rosettes of soil-grown plants (12 h photoperiod) at the end of the day (ED) and the end of the night (EN). Note the reduced staining of 35S:ATAF1 rosette at ED and EN. Data in graphs represent means ± SD of three biological replicates with 10 seedlings each. Asterisks indicate significant difference to controls (p < 0.05; Student’s t-test).

Figure 4. Gene Expression and Sugar Levels. A, Heat map of expression of KIN10-regulated genes in shoots of ATAF1 transgenic seedlings compared to WT. Gene expression analysis was performed on shoots of seedlings grown in trehalose (25 mM) medium for 10 days. B, Heat map of gene expression determined by qRT-PCR in seedlings (left), or detached leaves of 3-week-old soil-grown plants subjected to dark incubation on moist filter paper for 3 days (right). Differences to controls (mock treatment for ATAF1-IOE, and WT for 35S:ATAF1 and ataf1-4 plants) are shown. Data in (A) and (B) are means of three biological replicates, each determined in two technical replicates. C, Sugar levels in seedlings grown for two weeks on one-half strength MS + 1% (w/v) sucrose medium. Means ± SD (three replicates with 10 seedlings each). Asterisks indicate significant difference from controls (p < 0.05; Student’s t-test). Full data for (A) and (B) are given in Supplemental Table I.

Figure 5. Number of Carbon Fixation-responsive Genes Differentially Expressed in ATAF1-IOE upon ESTR Induction. A, Genes repressed by carbon fixation that are induced in ATAF1-IOE. Note, that the number of carbon fixation-repressed genes that are induced in ATAF1-IOE increases with extended ESTR induction times. B, Genes induced by carbon fixation that are repressed in ATAF1-IOE. Note that the number of carbon fixation-induced genes that are repressed in ATAF1-IOE increases with extended ESTR induction times. Carbon fixation-responsive genes were taken from Bläsing et al. (2005). Fch, fold change.

Figure 6. Number of Carbon/Energy Status-responsive Genes Differentially Expressed in ATAF1-IOE upon ESTR Induction. A, Genes up- or downregulated by at least 2-fold in
various carbon re-addition experiments (numbered 1 – 4; Bläsing et al., 2005; Osuna et al., 2007) and high energy-status experiments (numbered 5 – 7; Thimm et al., 2004; Bläsing et al., 2005) were compared with genes induced after 5 h of ESTR induction in leaves of 30-day-old *ATAF1-IOE* plants. Note that the vast majority of the genes repressed by carbon resupply/high energy status were induced in *ATAF1-IOE*. B, The genes up- or downregulated by at least 2-fold in the various carbon-starvation experiments were compared with genes repressed after 5 h of ESTR induction. Note that the vast majority of the genes induced by carbon resupply/high energy status were repressed in *ATAF1-IOE*. C, Genes affected by extended darkness (Usadel et al., 2008) and expressed differentially in *ATAF1-IOE*. *pgm*, phosphoglucomutase mutant; ED, end of the day; EN, end of the night; XN, extended night; Fch, fold change.

**Figure 7: Model of ATAF1 Action.** Reduced carbon availability triggered by environmental input (e.g., extended darkness, low atmospheric CO₂ concentration, drought) induces *ATAF1* expression, while high expression of ATAF1 itself causes a low-carbon physiological status, suggesting the existence of a carbon starvation feed-forward loop that involves ATAF1 as a regulatory node. ATAF1 directly upregulates expression of *TRE1* by binding to its promoter, and additionally affects the expression of many other carbon starvation-related genes, directly or indirectly (indicated by a boxed question mark). X and Y indicate unknown mechanisms by which low carbon (potentially low sucrose) inhibits TPS and/or activates TPP, respectively (Yadav et al., 2014). Solid lines indicate direct regulatory interactions or metabolite conversions, while dashed lines indicate interactions of unknown molecular mechanism. Arrow- and T-ending lines represent activation and repression, respectively. ATAF1 may also lead to transcriptional reprogramming by virtue of the lowered Tre6P level it induces and a concomitant activation of SnRK1 activity (indicated as “SnRK1 regulation”) as suggested by other authors (Zhang et al., 2009; Nunes et al., 2013).

**SUPPLEMENTARY DATA**

**Supplemental Figure 1. Starch Content in Rosettes of Soil-grown Plants.** Plants were grown in a 12-h photoperiod and were 35 days old upon rosette harvest. Data are means ± SD of three biological replicates with three rosettes each. ED, end of day; EN, end of night. Asterisks indicate significant difference to WT (*p < 0.05; Student’s *t*-test).
Supplemental Figure 2. Number of KIN10 Target Genes Differentially Expressed in *ATAF1-IOE* Plants upon ESTR Induction. **A,** Genes induced in *KIN10* overexpressors that are upregulated in *ATAF1-IOE.* **B,** Genes repressed in *KIN10* overexpressors that are downregulated in *ATAF1-IOE.* Note the increasing coincidence of *KIN10-* and *ATAF1-* mediated transcriptional changes upon extended ESTR induction times in *ATAF1-IOE* plants. KIN10-affected genes were taken from Baena-Gonzalez (2007). **C,** Increased expression of *ATAF1* in 2-week-old *ATAF1-IOE* seedlings grown on one-half-strength MS medium containing 1% (w/v) sucrose and mature leaves from soil-grown plants treated with estradiol for the indicated times. *ATAF1* expression in ESTR-induced samples was compared to samples from mock-treated plants. Expression was determined by qRT-PCR. Data represent means ± SD of three independent biological replicates (data for seedlings were taken from Supplemental Table I of Garapati et al., 2015). Fch, fold change.

Supplemental Figure 3. Expression of *ATAF1* is enhanced at low CO₂ concentration.
Expression of *ATAF1* in Arabidopsis plants illuminated for 4 h at ambient or low (< 50 ppm) CO₂ concentration (data extracted from Supplemental Table 6M of Bläsing et al., 2005).

Supplemental Figure 4. Clustering and GO annotation of genes differentially expressed after estradiol induction in *ATAF1-IOE* seedlings. **A,** Short Time-series Expression Miner (STEM) clustering of differentially expressed genes (with expression >0.5 and <-0.5 log2 Fch) at 1 h, 2 h and 5 h after estradiol treatment compared to mock treatment resulted in 15 temporal expression profiles. Numbers in profile boxes indicate the profile ID number. Profiles highlighted in color contain a statistically significant number of genes. Genes in each profile are listed in Supplemental Table VI. The STEM tool is available at http://www.cs.cmu.edu/~jernst/stem/. **B,** Profiles (13, 8 and 12) of genes upregulated over time. Major GO terms include ‘response to water deprivation’, ‘autophagy’ (profile 13), ‘galactolipid metabolic process’, ‘cellular response to phosphate starvation’ (profile 8) and ‘response to salicylic acid stimulus’, ‘protein amino acid phosphorylation’ (profile 12). **C,** Profiles (7, 2 and 3) of genes downregulated over time. Major GO terms include ‘regulation of programmed cell death’, ‘carbohydrate biosynthetic process’, ‘sulfur compound biosynthetic process’ (profile 7), and ‘pentose-phosphate shunt’, ‘starch biosynthetic process’ (profile 2). Genes in profile 3 may be preferentially associated with ‘phenylpropanoid biosynthetic process’ and ‘response to jasmonic acid stimulus’. In (B) and (C), genes were analyzed for their ontologies using AgriGO (http://bioinfo.cau.edu.cn/agriGO). Boxes...
indicate GO terms named by their GO ID, term definition and statistical information. The color of the boxes indicates the significance of the terms and the degree of color saturation is directly proportional to the enrichment level of the term.

**Supplemental Table I.** Data of heatmaps.

**Supplemental Table II.** Comparison of KIN10-regulated genes with genes affected in *ATAF1-IOE* line after ESTR treatment.

**Supplemental Table III.** Comparison of carbon fixation-regulated genes with genes affected in *ATAF1-IOE* line after ESTR treatment.

**Supplemental Table IV.** Comparison of carbon/energy status-responsive genes with genes affected in *ATAF1-IOE* line after ESTR treatment.

**Supplemental Table V.** Comparison of genes regulated by extended darkness with genes affected in *ATAF1-IOE* line after ESTR treatment.

**Supplemental Table VI.** STEM clusters of ATAF1-affected genes.

**Supplemental Table VII.** Primer sequences.
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Supplemental Figure 4A.

Clusters ordered based on number of genes and profiles ordered by significance (default)

STEM clustering (http://www.cs.cmu.edu/~jernst/stem/)
Supplemental Figure 4B.
Supplemental Figure 4C.
Supplemental Figure 4. Clustering and GO annotation of genes differentially expressed after estradiol induction in ATAF1-IOE seedlings. A, Short Time-series Expression Miner (STEM) clustering of differentially expressed genes (with expression >0.5 and < -0.5 log2 Fch) at 1 h, 2 h and 5 h after estradiol treatment compared to mock treatment resulted in 15 temporal expression profiles. Numbers in profile boxes indicate the profile ID number. Profiles highlighted in color contain a statistically significant number of genes. Genes in each profile are listed in Supplemental Table VI. The STEM tool is available at http://www.cs.cmu.edu/~jernst/stem/. B, Profiles (13, 8 and 12) of genes upregulated over time. Major GO terms include ‘response to water deprivation’, ‘autophagy’ (profile 13), ‘galactolipid metabolic process’, ‘cellular response to phosphate starvation’ (profile 8) and ‘response to salicylic acid stimulus’, ‘protein amino acid phosphorylation’ (profile 12). C, Profiles (7, 2 and 3) of genes downregulated over time. Major GO terms include ‘regulation of programmed cell death’, ‘carbohydrate biosynthetic process’, ‘sulfur compound biosynthetic process’ (profile 7), and ‘pentose-phosphate shunt’, ‘starch biosynthetic process’ (profile 2). Genes in profile 3 may be preferentially associated with ‘phenylpropanoid biosynthetic process’ and ‘response to jasmonic acid stimulus’. In (B) and (C), genes were analyzed for their ontologies using AgriGO (http://bioinfo.cau.edu.cn/agriGO). Boxes indicate GO terms named by their GO ID, term definition and statistical information. The color of the boxes indicates the significance of the terms and the degree of color saturation is directly proportional to the enrichment level of the term.


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