

Research Article

Comparative Transcriptomic Analysis for Wheat Etiolated and Green Seedlings during Vernalization

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Abstract

Light can promote the growth and development of wheat seedling during vernalization. However, the mechanism has not been sufficiently explored. In this study, transcriptomic analysis was performed on wheat etiolated seedlings and green seedlings vernalized in dark and light, respectively. Results showed that light could promote photo morphogenesis and photosynthesis of wheat seedlings and increase fresh weight of seedlings, including root biomass and leaf area. The number of differentially expressed genes (DEGs) between etiolated and green seedlings increased with vernalization time increasing, and the maximum value occurred on vernalization 40 d. GO and KEGG enrichment analysis showed that the main DEGs on vernalization 40 d were significantly different from those on 10, 20 and 30 d in functions. Top GO and KEGG enrichment related to photosynthesis, such as chloroplast, thylakoid, photosynthesis and oxidoreductase activities, were identified on vernalization 40 d. Then a series of candidate genes were identified, 15 of which were confirmed by qRT-PCR. The above findings provide valuable information for understanding the molecular mechanism of light on wheat seedlings during vernalization.

Keywords: Winter wheat, Vernalization, Seedlings, Light, Photosynthesis

Introduction

Wheat (*Triticum aestivum* L.) is one of the most widely cultivated crops in the world. Its growth and development are determined by complex genetic and environmental factors, in which genes related to vernalization and photoperiod sensitivity play more important roles [1,2].

Winter wheat is sensitive to vernalization and requires a period of continuous cold temperature for its transition from vegetative to reproductive growth [3]. The vernalization response is also integrated with the environmental cue of light. VRN3 is an integrator of the vernalization and photoperiod pathways in temperate cereals, the role of which has been well documented [1,4]. Plants have a sophisticated network to decipher the information of light-temperature integration. The sensation can be defined as a process in which a sensory receptor changes its activity as a result of a stimulus. The photoreceptors phyB and phot have been identified as temperature sensors [5-7].

Light is one of the major environmental factors that regulate seedling growth and development. After seed germination under the soil, dark-grown seedlings usually grow heterotrophically from seed reserves and accumulate protochlorophyllide (Pchl_{id}) via a process referred to as skotomorphogenesis (etiolation; [8]). Upon exposure to light, Pchl_{id} is rapidly converted into chlorophyll to initiate photoautotrophic growth, resulting in large-scale genes expression of photomorphogenesis (greening) [9]. Photomorphogenesis is

marked by chlorophyll biosynthesis, differentiation of proplastids into chloroplasts, initiation of carbon assimilation, elongation and thickening of hypocotyl, and the activation of shoot apical meristem leading to the development [10,11]. There is a comprehensive regulatory network that corresponds to specific morphological aspects in seedling skotomorphogenesis and photomorphogenesis [12-14].

The vernalization in light can promote winter and semi-winter wheat varieties flowering earlier than in dark for [15-17], when plants are stimulated by two external signals, light and low temperature. Some vernalization-responsive genes or proteins have been identified during wheat vernalization by high-throughput transcriptomics and proteomics [18,19]. However, the extent to which genes contribute to wheat seedling development during vernalization remains unclear.

In this study, we conducted RNA-seq analysis to determine the effect of light on transcriptomic changes of wheat seedlings during vernalization. Further, the hypothetical molecular mechanisms of several genes responding to vernalization of wheat in light were discussed.

Materials and Methods

Plant Materials and Vernalization

The uniform seeds of winter wheat Liangxing 99 (vrn-A1/vrn-B1/vrn-D1) were selected and cultivated on plates supplied with moist vermiculite at 25°C in dark, 30 seeds per plate. After 3 d, the developed

seedlings were vernalized in dark or in continuous light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) in a growth cabinet at 6°C . The light was provided by cool white fluorescent lamp. At each timepoint of vernalization 0, 10, 20, 30 and 40 d, the fresh weight of single plant, fresh weight of seedling leaves, fresh weight of roots, and the longest root length were all measured. Three biological replicates were conducted. SPSS19.0 and Sigmaplot 12.0 were performed to analyze the difference between etiolated and green seedlings.

RNA-seq Sample Preparation and Sequencing

At each investigation timepoint, about 500 mg samples of shoots and leaves per treatment were collected from 5-15 seedlings for total RNA isolation (Figure 1a and 1b). When sampling, the collected leaves and shoots were immediately frozen in liquid nitrogen and then stored at -80°C . Total RNA was extracted with TRIzol™ Reagent (ThermoFisher, USA) following manufacturer's instructions and confirmed using the 2100 Bioanalyzer (Agilent Technologies). mRNA was purified and then constructed into sequencing library using TruSeq RNA Sample Preparation Kit v2 (#RS-122-2001, Illumina, USA) following manufacturer's recommendations. The obtained libraries were adjusted and pooled at a concentration of 20 nmol. Sequencing was performed with 300-400 bp, paired-end reads on the NextSeq 500 (Illumina). Each treatment had two biological replicates.

Data Processing, De-novo Transcriptome Assembly, Differential Expression Gene Analysis

Clean reads with Q-score >20 was further analyzed by removing the adapters and filtering low Q-score reads from raw reads. Reference genome index was constructed using Bowtie2 Software (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>) based on the data in Ensembl (<http://www.ensembl.org/>). The clean reads were aligned to the reference genome by Tophat2 (<http://tophat.cbcb.umd.edu/>). The original expression level of corresponding gene was considered to be the alignment value (read count) and was analyzed by HTSeq0.6.1p2 (<http://wwwhuber.embl.de/users/anders/HTSeq/>). The read count was normalized with reads per kilobases per million mapped (RPKM), with >1 as gene expressing threshold. Furthermore, the differential expression genes (DEGs) were analyzed by DESeq with $|\text{fold change}| > 2$, P-value < 0.05 , and false discovery rate (FDR) < 0.05 .

Functional Analysis on DEGs

Gene ontology (GO, <http://geneontology.org/>) terms with corrected P-value of less than 0.05 were considered to be significantly enriched. KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.kegg.jp/>) enrichment pathways of DEGs were confirmed with P-value <0.05 . The DEGs related to photosynthesis and having a minimum 3-fold change were further analyzed.

Evaluation of RNA-Seq by qRT-PCR

To verify the reliability and accuracy of our transcriptomic data, 15 DEGs associated with photosynthesis, signaling interaction, amino acids metabolism, fatty acid metabolism, etc. were selected and evaluated by qRT-PCR. The primers designed with Primer 5.0 Software (PREMIER Biosoft, USA) are detailed in Table S1. Actin was used as an internal

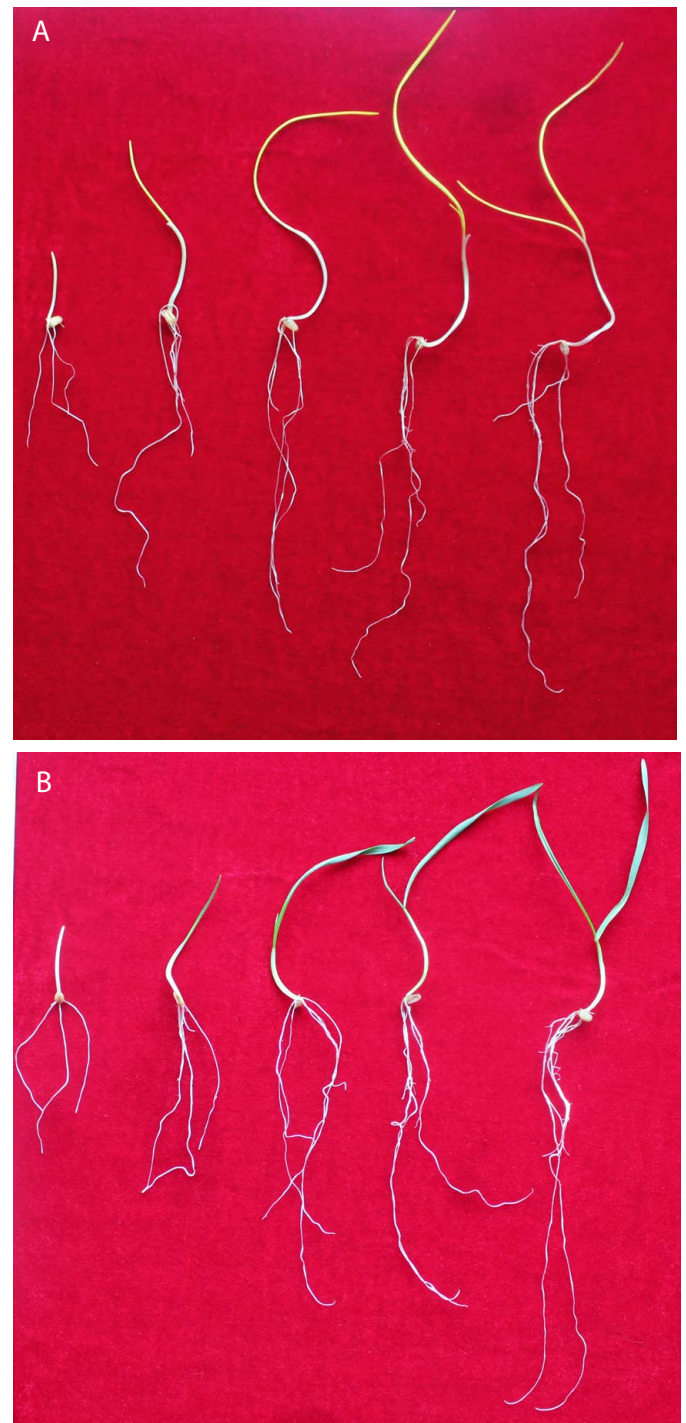


Figure 1: Etiolated and green wheat seedlings used for generating transcriptome, which were vernalized in dark and in light respectively. On each panel, seedlings from left to right were vernalized for 0, 10, 20, 30, and 40 d, respectively.

control [20]. Using the identical RNA samples for RNA-seq, qRT-PCR was performed on a C1000 Thermal Cycler (CFX96 Real-Time System, Bio-Rad, USA) using a Quant One Step RT-PCR (SYBR Green) Kit (TianGen, China) following manufacturer's instructions. Three independent biological replicates and three technical replicates per biological replicate were conducted. The PCR efficiency of target and reference genes were determined by generating standard curves and the

relative expression values were calculated using the $2^{-\Delta\Delta CT}$ method [21]. With the expression level value on 0 d set as 1, each DEG expression level was recorded and its significance was analyzed by the independent t-test at P -value <0.05 . In addition, the relative expression levels of DEGs were analyzed based on RNA-Seq and qRT-PCR, respectively.

Results

Performance of Wheat Seedlings Vernalized in Dark and in Light Conditions

At the beginning, the seedlings for vernalization treatments were exactly the same. On vernalization 10 d, etiolated seedlings,

which was vernalized in dark, showed significantly higher fresh weight (including single plant fresh weight, shoot fresh weight and root fresh weight) and root length than green seedlings (Figure 1). However, with the initiation of the photosynthesis and photomorphogenesis, the fresh weight of green seedlings (vernalized in light) were gradually increased and became more than that of etiolated seedlings from vernalization 20 d (Figure 2A). Although green seedlings had less shoot fresh weight compared with etiolated seedlings on vernalization 40 d (Figure 2B), their root fresh weight and length were both significantly more than etiolated seedlings. This indicated that light promoted the growth of wheat seedlings during vernalization (Figure 2C and 2D).

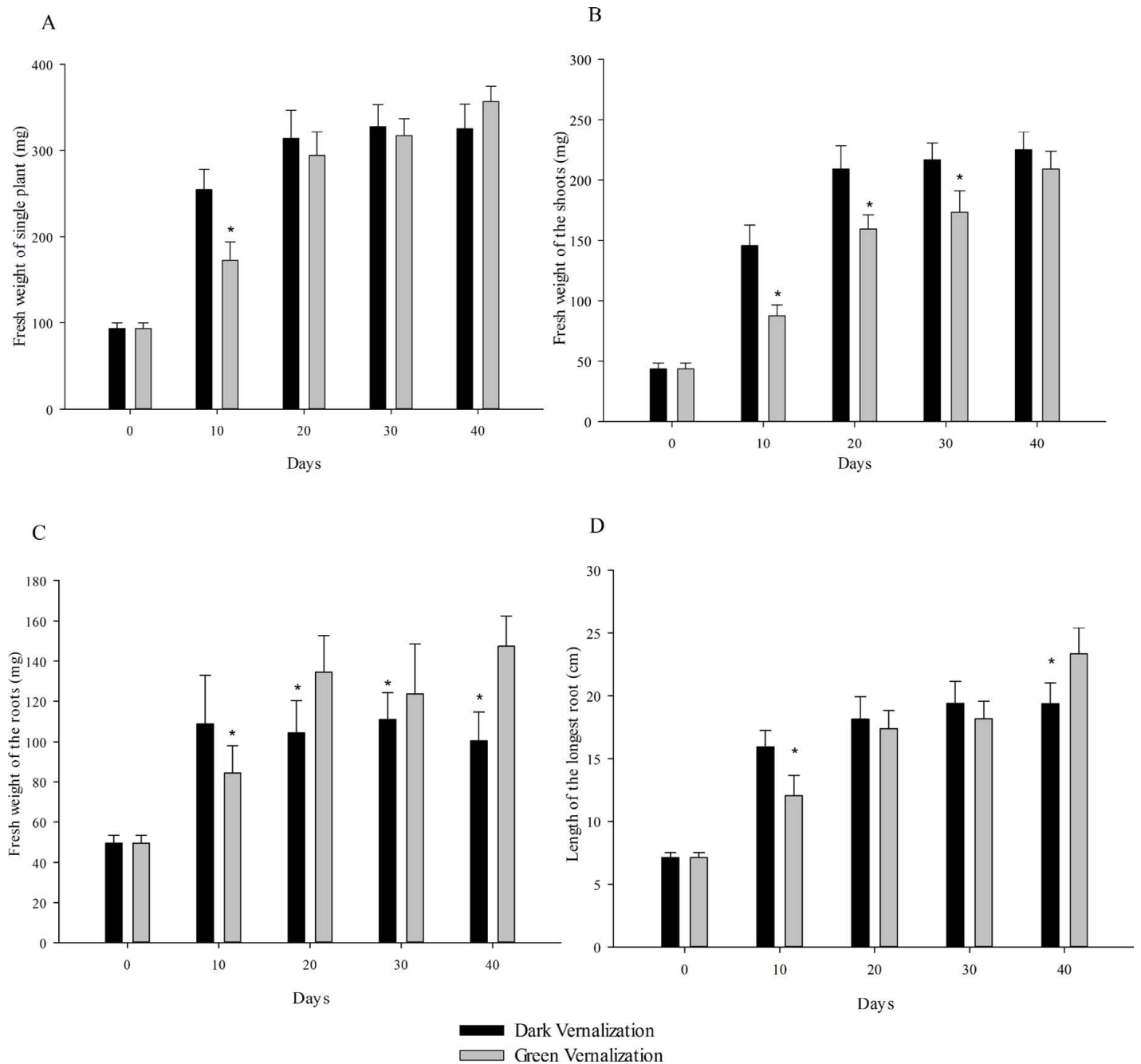


Figure 2: Performance of wheat seedlings development at five timepoints (0, 10, 20, 30, and 40 d) under dark and light vernalization treatments. A: fresh weight of single plant; B: fresh weight of shoots; C: fresh weight of roots; D: length of the longest root. *, data are mean \pm SD of three replicates, P -value <0.05 .

Identification of Differential Expressed Genes

The expression level of the DEGs was calculated and normalized to FPKM (fragments per kilobase million), and then DEGs between etiolated and green seedlings were screened with fold change >2 and P-value <0.05. The number of DEGs between etiolated and green seedlings on vernalization 0, 10, 20, 30 and 40 d were 0, 6131, 6971, 7249, and 11807, respectively (Table 1). The maximum was on vernalization 40 d, indicating that more metabolic activities had occurred in green seedlings.

Evaluation of RNA-Seq Data by qRT-PCR

To verify the RNA-seq results, 15 DEGs on vernalization 40 d were further analyzed by qRT-PCR. The expression level of every DEG in etiolated seedlings was set as 1. Then their expression level in green seedlings were recorded. qRT-PCR and t-test analysis showed that each of the 15 DEGs showed significantly different expression levels between etiolated and green seedlings, which was consistent with the result from transcriptome sequencing. Also, their expression patterns correlated well ($R^2 = 0.8123$) with those obtained from RNA-seq analysis (Figure 3). Therefore, the transcriptome changes obtained by RNA-seq were accurate.

GO Enrichment Analysis of DEGs

The functions of DEGs were annotated by GO enrichment analysis (P-value <0.05) and then they were divided into three terms: biological process, cellular component and molecular function. The topGO enrichment results for DEGs on 10, 20 and 30 d were almost the same. The biological processes of DEGs were mainly involved in cell wall macromolecule catabolic process, aminoglycan catabolic process, chitin catabolic process and oxidation-reduction process (Figures S1-S3); the cellular components were mainly extracellular region, cytoplasmic region, endoplasmic reticulum, photosystem and membrane part

(Figures S4-S6); the molecular functions were mainly involved in oxidoreductase activity, chitinase activity, catalytic activity, heme binding, iron ion binding and tetrapyrrole binding (Figures S7-S9).

On vernalization 40 d, a total of 2180 DEGs between etiolated and green wheat seedlings were annotated by GO enrichment analysis (P<0.05). The biological process was mainly involved in photosynthesis, chloroplast organization, cellular ketone biosynthesis process (Figure S10), the cell components were associated with chloroplast, plastid, thylakoid (Figure S11), and the molecular function was associated with chlorophyll binding, fructose-bisphosphatase activity, catalytic activity (Figure S12). This result indicated that the DEGs between etiolated and green seedlings on vernalization 40 d were mainly related to photosynthesis.

KEGG Pathway Analysis of DEGs

KEGG enrichment pathways were selected by DESeq with [fold change] > 2, P-value <0.05, and false discovery rate (FDR) <0.05. The top KEGG pathway of DEGs on vernalization 10, 20 and 30 d were nearly the same. They were involved in amino acids metabolism, fatty acid metabolism, metabolism of cytochrome P450, photosynthesis, environmental adaptation, etc. (Figure 4a-4c). While on vernalization 40 d, the top KEGG enrichment pathways were mainly involved in photosynthesis (photosynthesis-antenna proteins, porphyrin and chlorophyll metabolism, photosynthesis, photosynthetic biological nitrogen fixation, carbon fixation in photosynthetic organisms, etc.), secondary metabolism (including synthesis of flavonoid, anthocyanin, ubiquinone and terpenoid-quinone biosynthesis, indole alkaloid, phenylpropanoid, riboflavin, etc.), nitrogen and carbon metabolism (including vitamins, amino acids, carbon, methane, glyoxylate and dicarboxylate, etc.) and environmental adaptation (circadian rhythm, longevity regulating pathway, and estrogen signal transduction, etc.) (Figure 4d).

Table 1: Primers for quantitative real-time PCR (qRT-PCR).

Gene ID	Forward primer (5'-3')	Reverse primer (5'-3')
TRIAE_CS42_4AL_TGACv1_289136_AA0965610	TGCGGGACAATATATCTCA	GTGAGGATCAAACACATCG
TRIAE_CS42_7BL_TGACv1_579174_AA1904930	AGTGGTATGCCTGCGAGTGC	CTGCTGCTTGTGATGATTGC
TRIAE_CS42_5DL_TGACv1_432937_AA1395210	CGATGCCAACAGCGACAA	CGCCATTGATACCCGCTCTT
TRIAE_CS42_3AL_TGACv1_195180_AA0645990	GCTACGCACTTTACGGTATCACA	ACCTTCGCCAACTCCTTCTC
TRIAE_CS42_2DS_TGACv1_179360_AA0606510	CATCATGGAGGGAGAAACCG	CCGCATACTTGCCAAACCTG
TRIAE_CS42_3B_TGACv1_237526_AA0832830	GGAGCTGGTCGAGTTGAAGA	GAACCAAGCCGCTATCTGGT
TRIAE_CS42_1BS_TGACv1_052086_AA0181320	ACCTGGTGTGCGCATAGAAT	CGTTGGGTCGTCAAACCTCATA
TRIAE_CS42_2BL_TGACv1_727308_AA2170970	ACTATCACCCATCGCATCACA	ACACGCCTTCCATTCTCCC
TRIAE_CS42_3AL_TGACv1_195180_AA0645980	CGGGCCTCGCAATTTACA	AGTCTCGCCAACTCGGTCT
TRIAE_CS42_5DL_TGACv1_433817_AA1422930	ACCGCCAAACAAACCCAA	GCAGTCACGAAACCCACCAT
TRIAE_CS42_6AS_TGACv1_486935_AA1566680	ACAGCACCAACTACTGCATCC	GGAAGAAGACGACCATCTCCA
TRIAE_CS42_6BS_TGACv1_514208_AA1656980	TCGAGGGGTACTGCATTGTC	CTTGATCTCCTTCACTTGAGC
TRIAE_CS42_7BS_TGACv1_594457_AA1957650	TACCGACTTCTGCTTCCACTCA	ACCCAGTAATCATCAATACATCC
TRIAE_CS42_5DL_TGACv1_433244_AA1406760	GTTCTACACGCCGACAAGA	GGTAGCGGTGATAGGGTTT
TRIAE_CS42_6BL_TGACv1_505061_AA1629060	CGGACTGGTTCAGGAAGGAC	CACGTGATGTGAGGGTAGGC
Actin	GAAGCTGCAGGTATCCATGAGACC	AGGCAGTGATCTCCTTGCTCATC

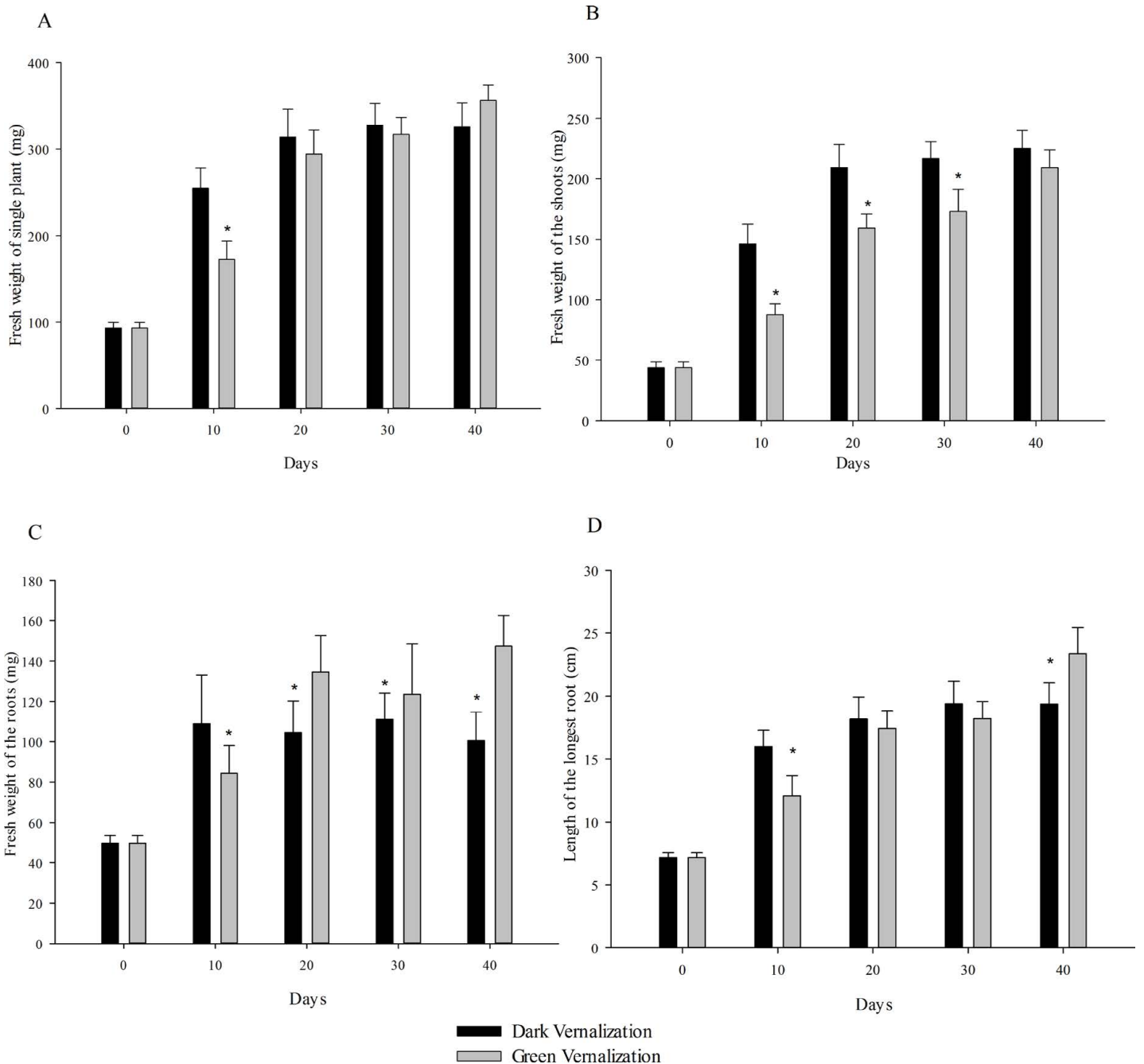


Figure 3: Correlation analysis of the 15 differentially expressed genes (DEGs)’ expression levels revealed by RNA-seq and qRT-PCR, respectively. The plots indicate the log₂ (Fold change) in RNA-seq and qRT-PCR.

DEGs Related to Photosynthesis during Wheat Vernalization

To further understand the effect of light on photosynthesis during vernalization, we focused on the DEGs with a minimum three-fold change in expression between etiolated and green seedlings at V40 d according to KEGG enrichment analysis. Compared with etiolated seedlings, the up-regulated genes in green seedlings were related to chloroplast component, nitrogen and carbon synthesis, while the down-regulated genes were related to catalase, dehydrogenase, ubiquitin, phosphate dikinase and glutamine synthetase.

Discussion

The effect of light on the morphological development of seedlings is achieved through regulating the expression levels of a series of genes [22]. In this study, we used whole-transcriptome sequencing to analyze the seedlings vernalized in dark and in light conditions to identify their molecular changes at different times of vernalization. The results showed that light can regulate a series of genes related to skotomorphogenesis and photomorphogenesis, which is consistent with previous studies [11,12,22,23].

Usually, either triggering photosynthesis or initiating flowering is the acting mode of light on plants. The former is the energy source of plant, while the latter is the developmental key from vegetative growth to reproductive development [24,25]. Interestingly, the results in this paper indicated that light can significantly increase the length and fresh weight of roots but inhibit the growth of above ground part of seedlings. Previously, it has been proved that light influence root development and plasticity through complex signaling pathways [26]. Generally, the roots of dark-grown seedlings are much shorter and have a much thinner diameter than those of light-grown seedlings [27,28]. In speed breeding, green plant vernalization can promote winter wheat flowering earlier in the subsequent development process [16,17]. Therefore, during vernalization process, supplementation of light may be conducive to promoting the morphological development of roots and leaves, as well as indirectly promoting later flowering development.

In our study, GO and KEGG enrichment analysis shows that the significantly DEGs between seedlings vernalized in dark and in light are mainly involved in photosynthesis with the elongation of wheat vernalization. Chlorophyll is essential for harvesting light energy during photosynthesis. It is proved that the complex integration of intracellular chloroplast retrograde redox signaling combined with intercellular, and vascular-mediated signaling pathways during vernalization process enhance both photosynthetic capacity and plant biomass production in cold-grown winter cereals.

In this experiment, the level of chloroplast, photosystem II 10 kDa polypeptide, fructose 1,6-bisphosphatase, pyruvate, phosphate dikinase (PPDK) was higher in green seedlings than in etiolated seedlings. Chlorophyll is essential for light harvesting and energy transduction during photosynthesis. Leaf color results from the processes of chlorophylls accumulation in leaf, which is related to chloroplast development and division, biosynthesis [29]. Photosystem II (PSII) is a multisubunit protein-pigment complex embedded in the thylakoid membrane that harnesses light energy to split water into oxygen, protons, and electrons [30]. Fructose 1,6-bisphosphatase is required for optimum regulation of photosynthetic carbon metabolism. Fructose 6-phosphate (F6P) is the branch point for metabolites leaving the Calvin cycle and moving into starch biosynthesis through the conversion into glucose 6-phosphate (G6P) [31]. Pyruvate, phosphate dikinase (PPDK), a key role in the C4 photosynthetic pathway, proposed first functionally seated in C3 plants as an ancillary glycolytic enzyme [32]. In intact spinach chloroplasts, light-induced dephosphorylation of C(3) PPDK was shown to be dependent on photosystem II activity but independent of electron transfer from photosystem I [33-35].

Therefore, the above up-regulation genes associated with photosynthesis and Calvin cycle improves the biomass of seedlings under light. Meanwhile, the developing seedlings under dark could reduce their light-harvesting capacity and components of photosynthetic apparatus.

Conclusions

The analysis of DEGs between seedlings vernalized in dark and in light indicates that light influences plant growth and development

by triggering photosynthesis and feeding plants energy. Thus, the supplementation of light during wheat vernalization can directly improve the biomass of seedlings, as well as indirectly promote the later reproductive development.

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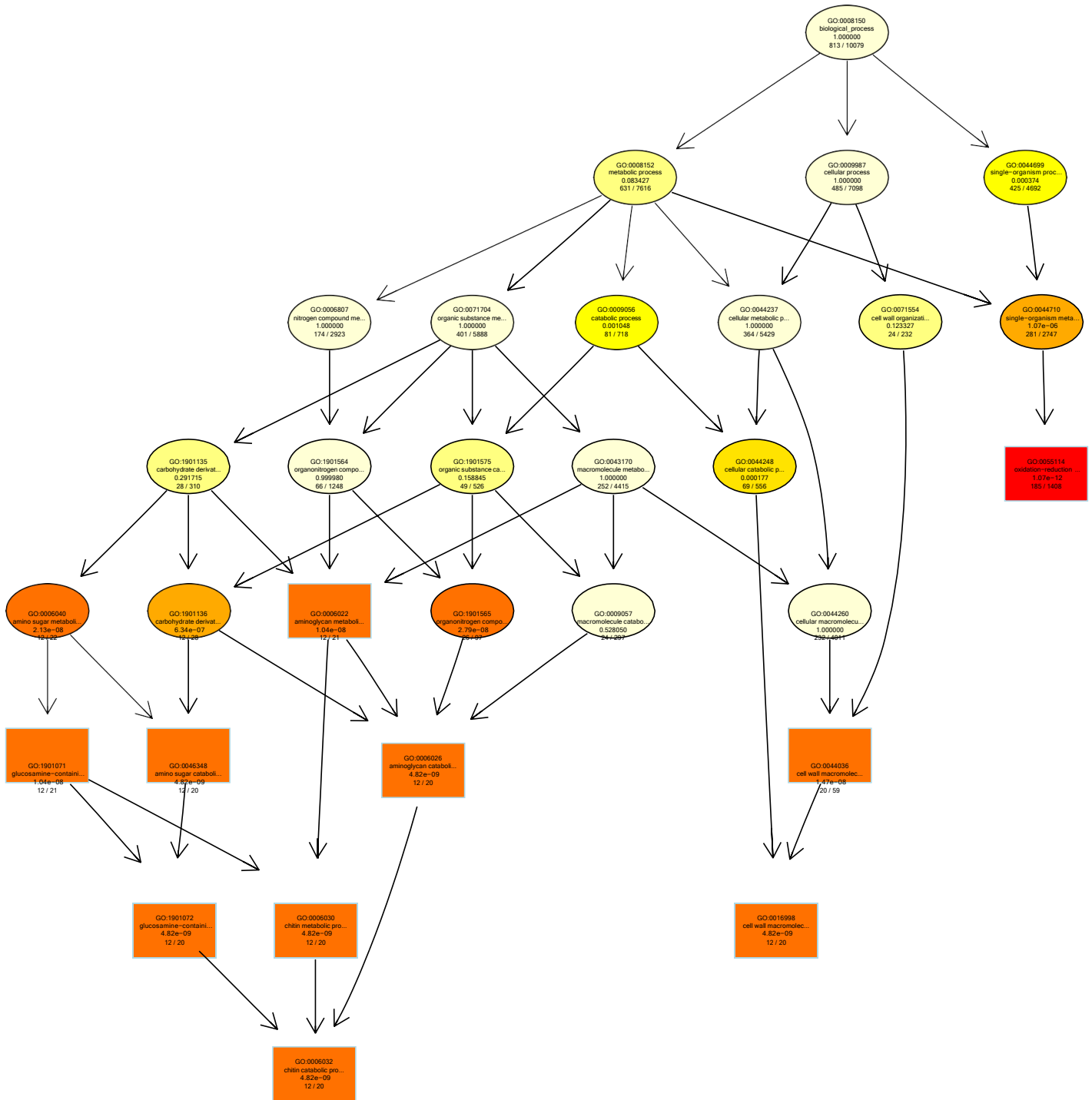


Figure S1: Biological processes that DEGs between etiolated and green wheat seedlings on vernalization 10 d involved in, revealed by topGO enrichment analysis.

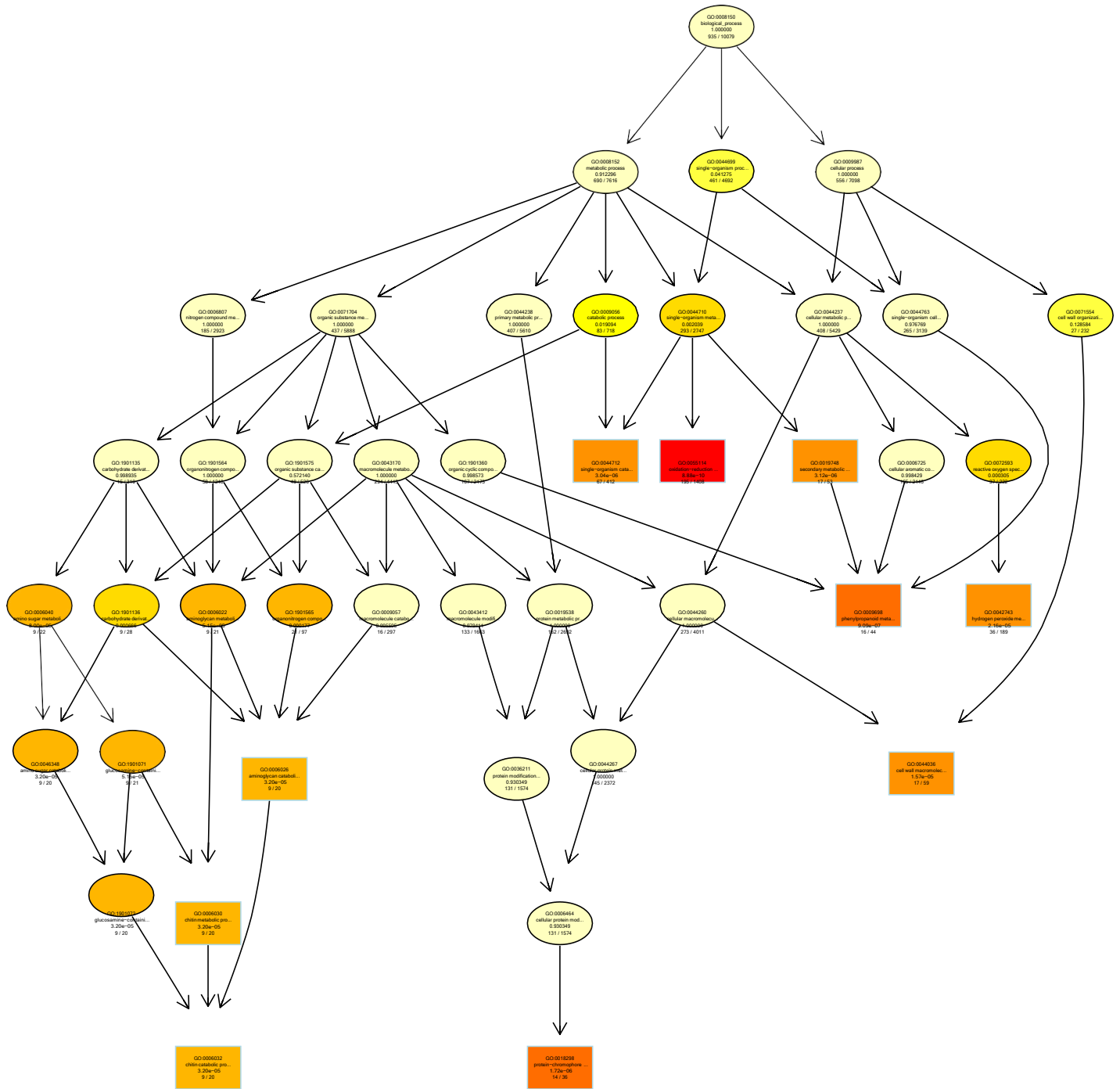


Figure S2: Biological processes that DEGs between etiolated and green wheat seedlings on vernalization 20 d involved in, revealed by topGO enrichment analysis.

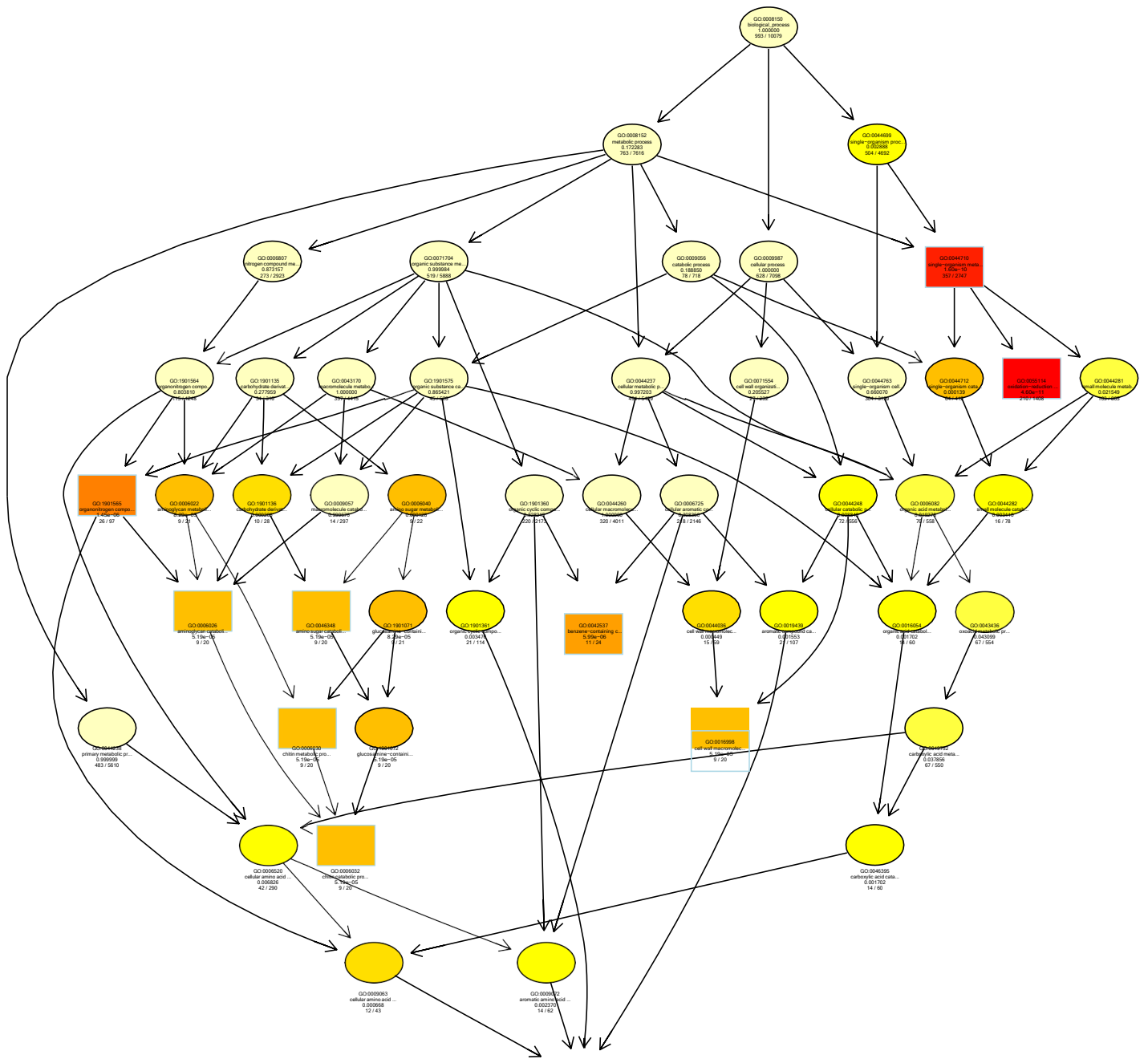


Figure S3: Biological processes that DEGs between etiolated and green wheat seedlings on vernalization 30 d involved in, revealed by topGO enrichment analysis.

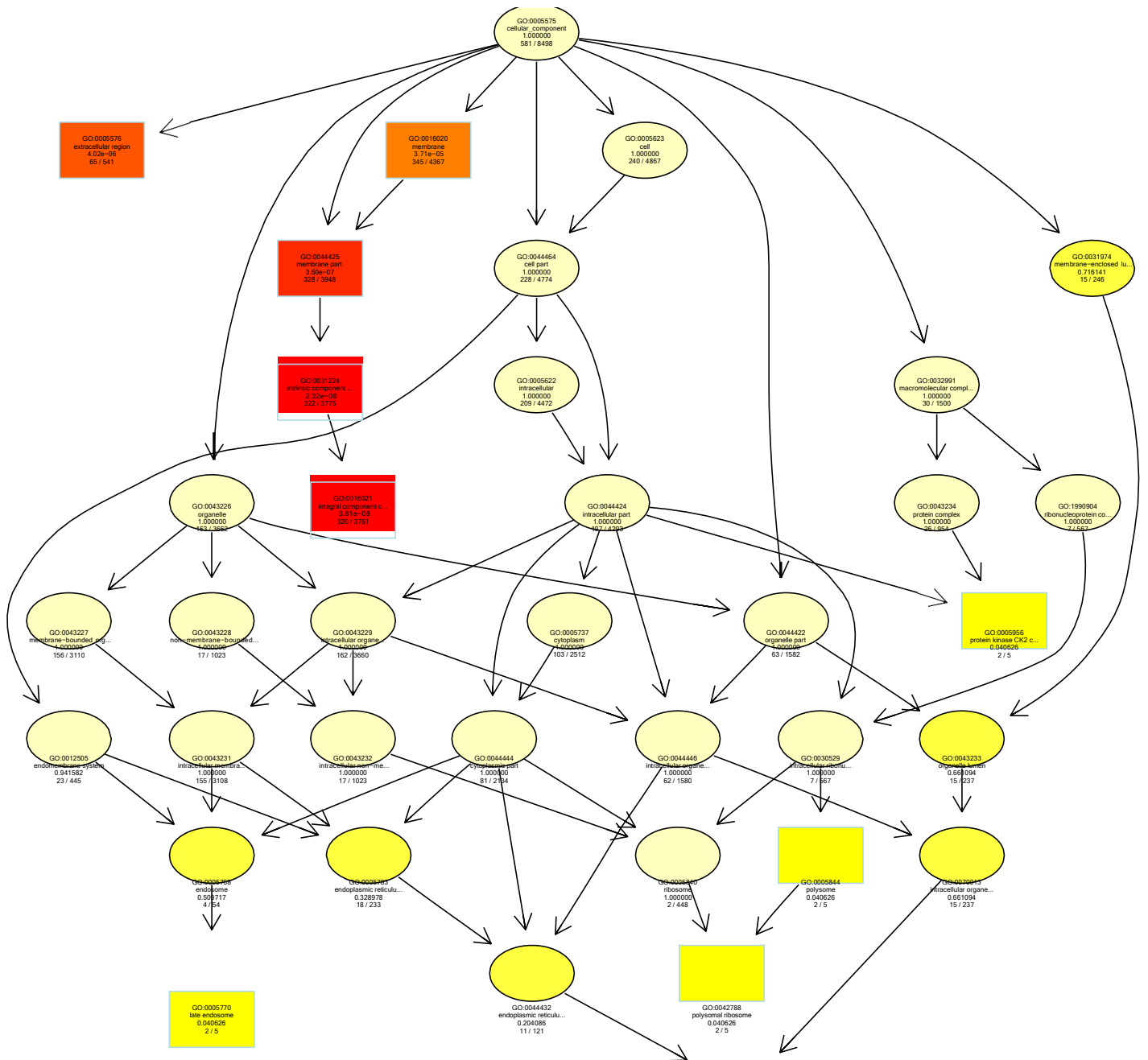


Figure S4: Cellular component where DEGs were found, revealed by topGO enrichment analysis. The DEGs were between etiolated and green wheat seedlings on vernalization 10 d.

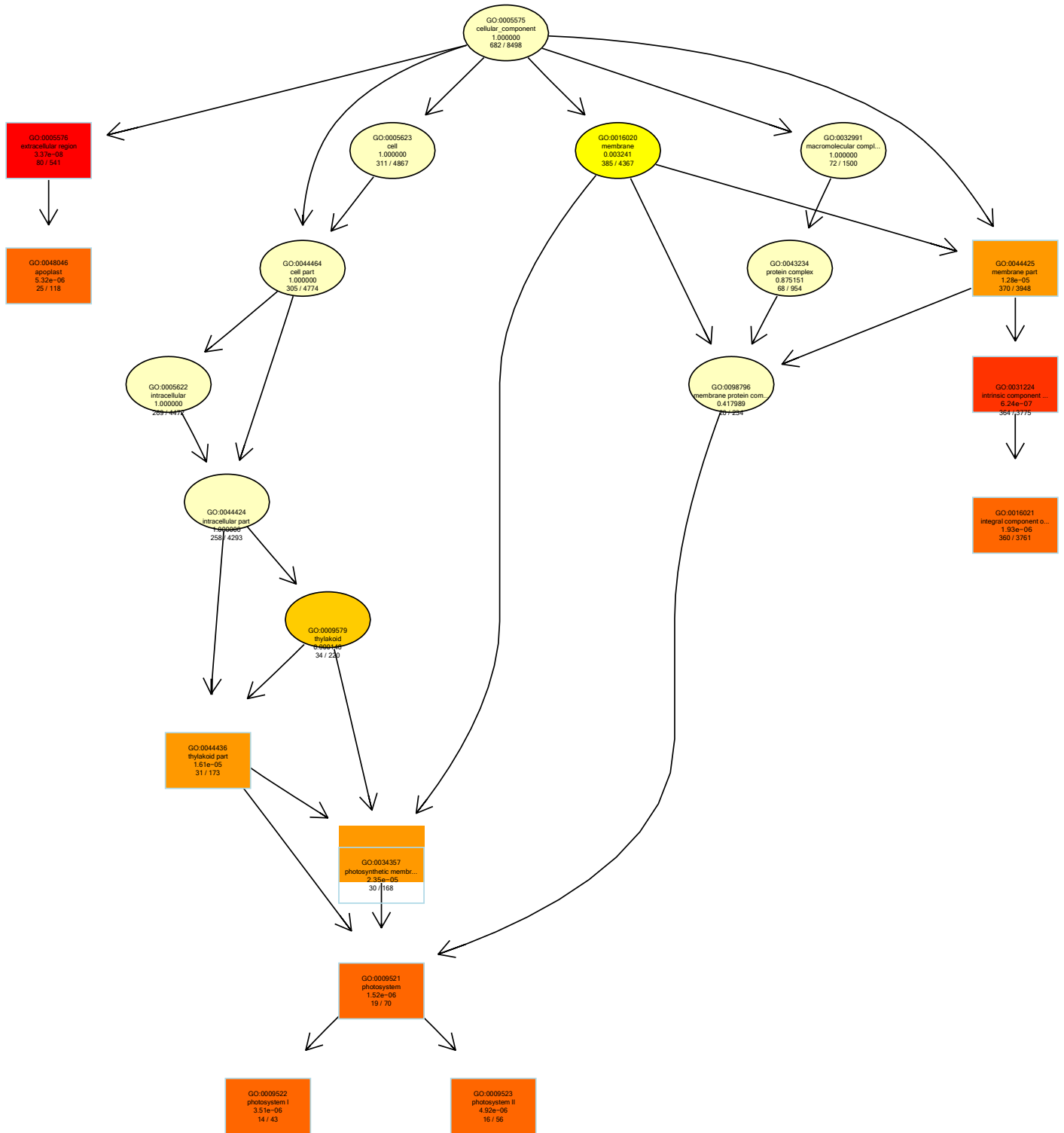


Figure S5: Cellular component where DEGs were found, revealed by topGO enrichment analysis. The DEGs were between etiolated and green wheat seedlings on vernalization 20 d.

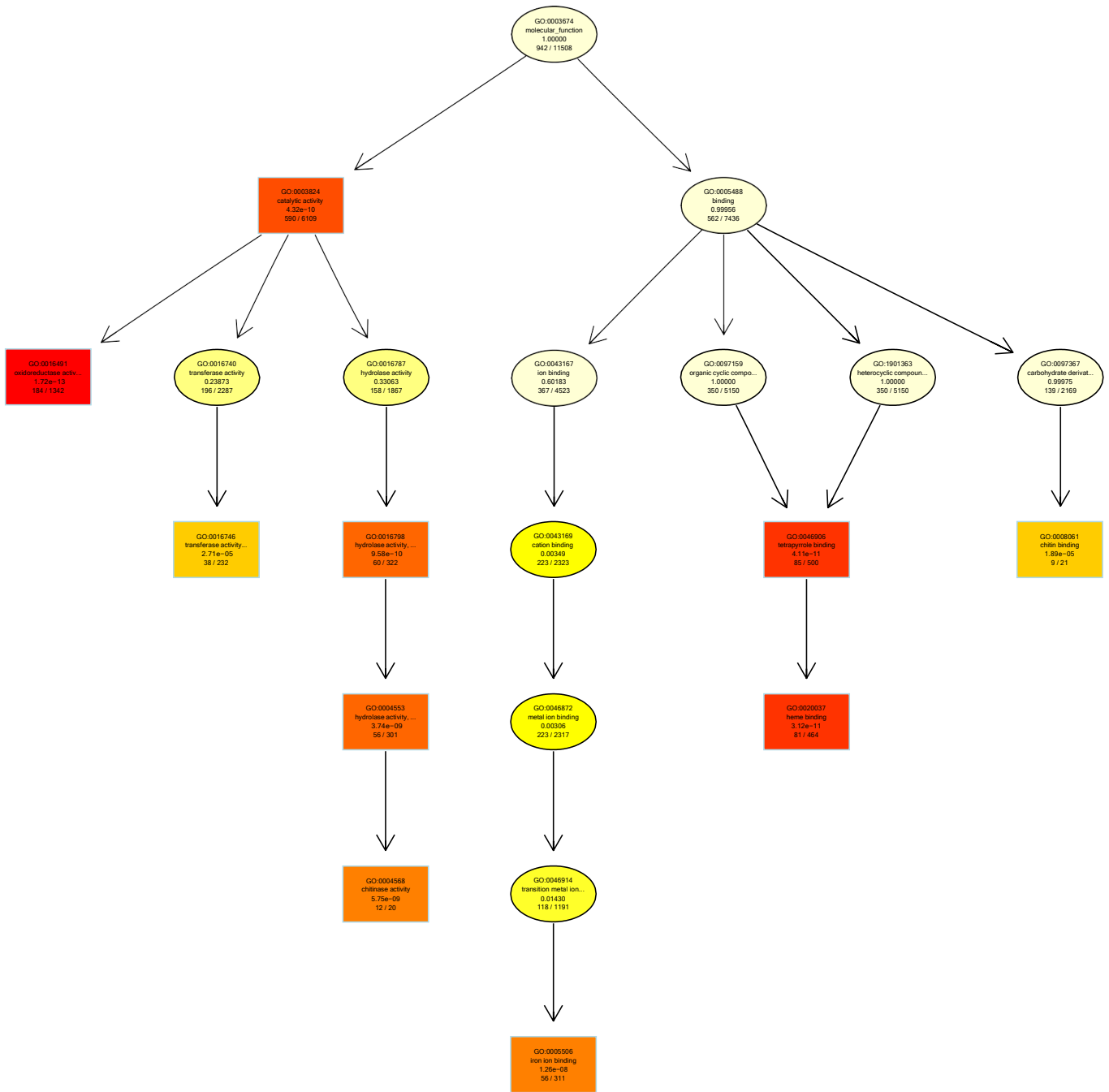


Figure S7: Molecular functions of DEGs between etiolated and green wheat seedlings on vernalization 10 d, revealed by topGO enrichment analysis.

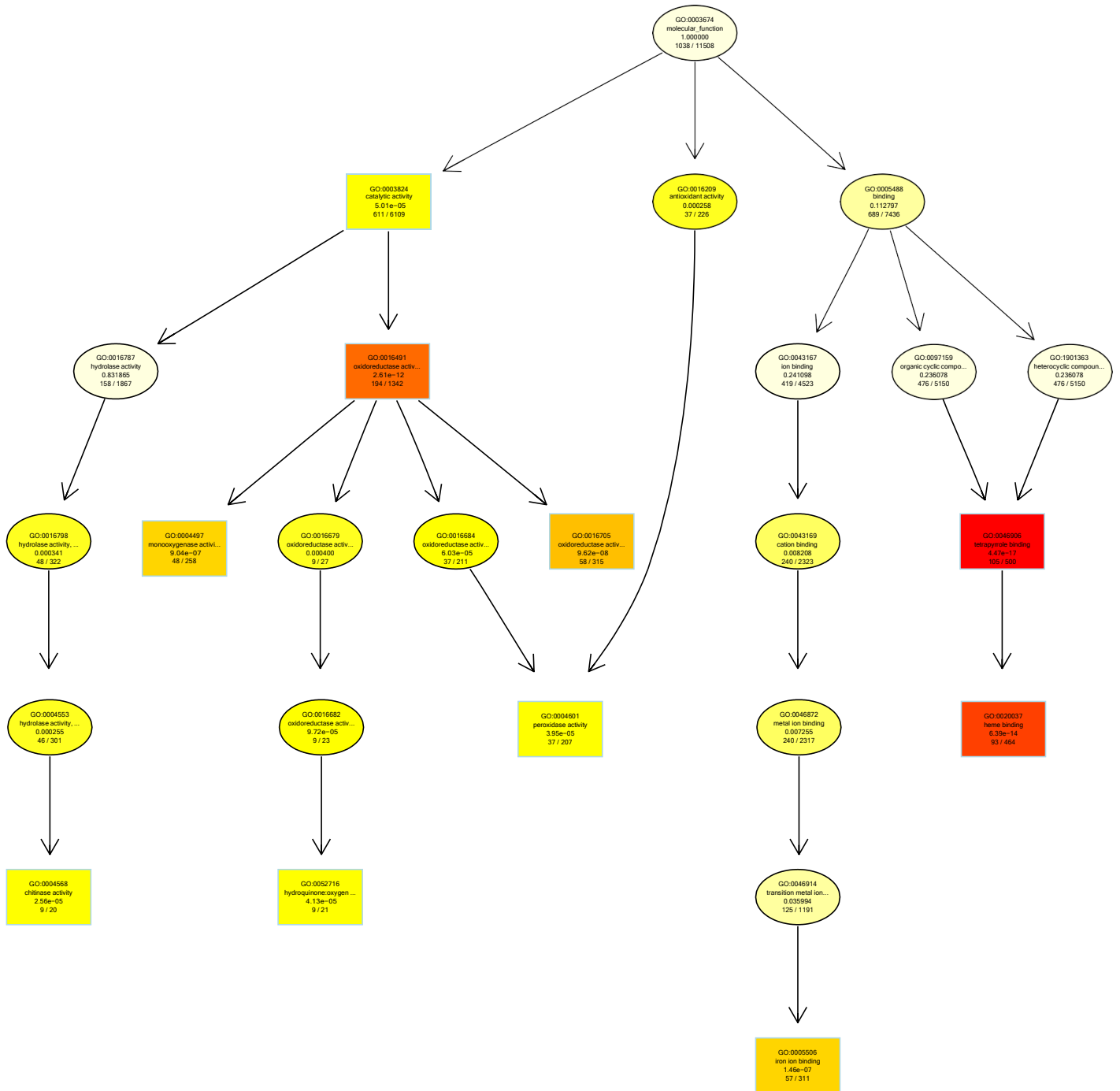


Figure S8: Molecular functions of DEGs between etiolated and green wheat seedlings on vernalization 20 d, revealed by topGO enrichment analysis.

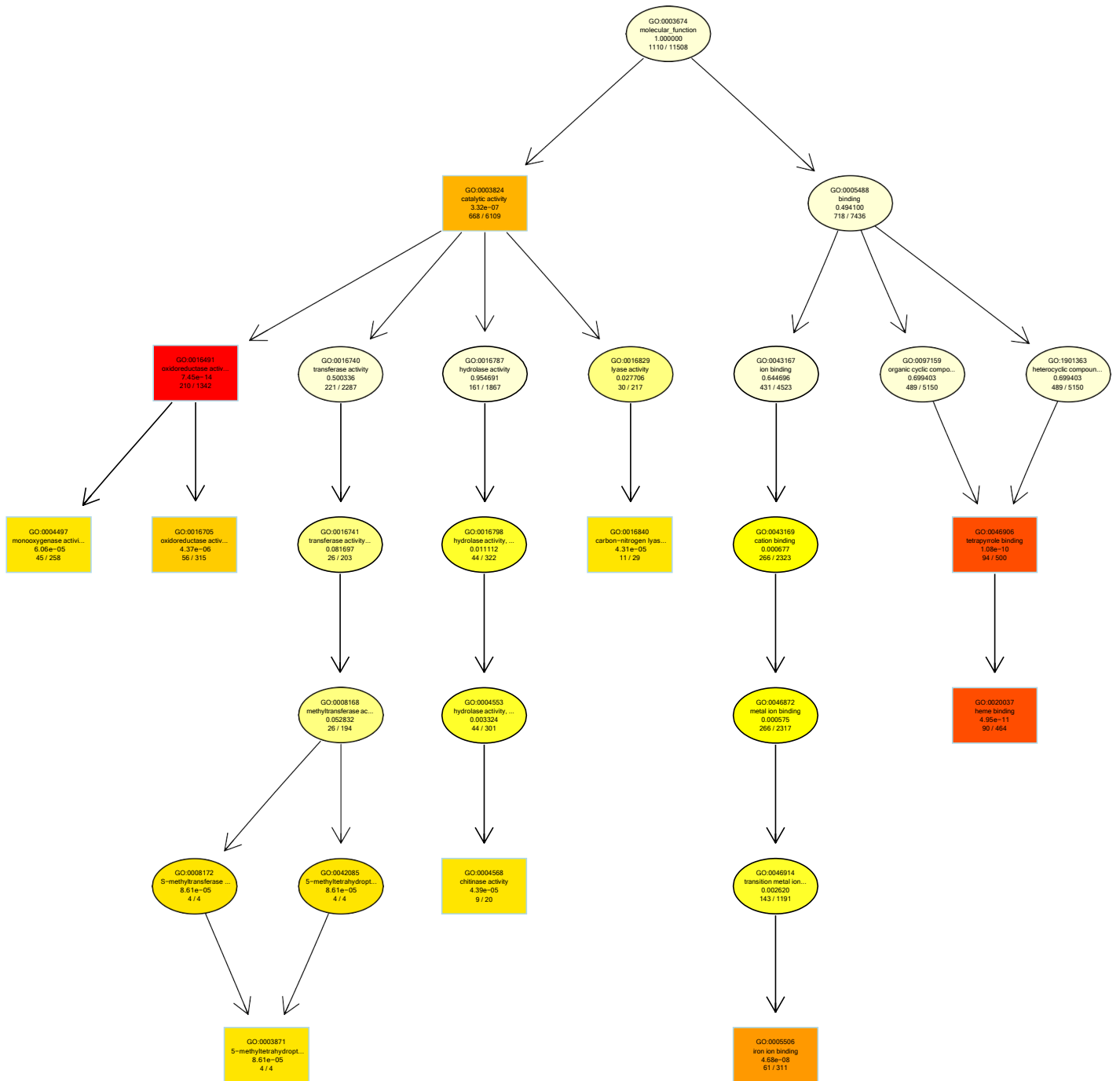


Figure S9: Molecular functions of DEGs between etiolated and green wheat seedlings on vernalization 30 d, revealed by topGO enrichment analysis.

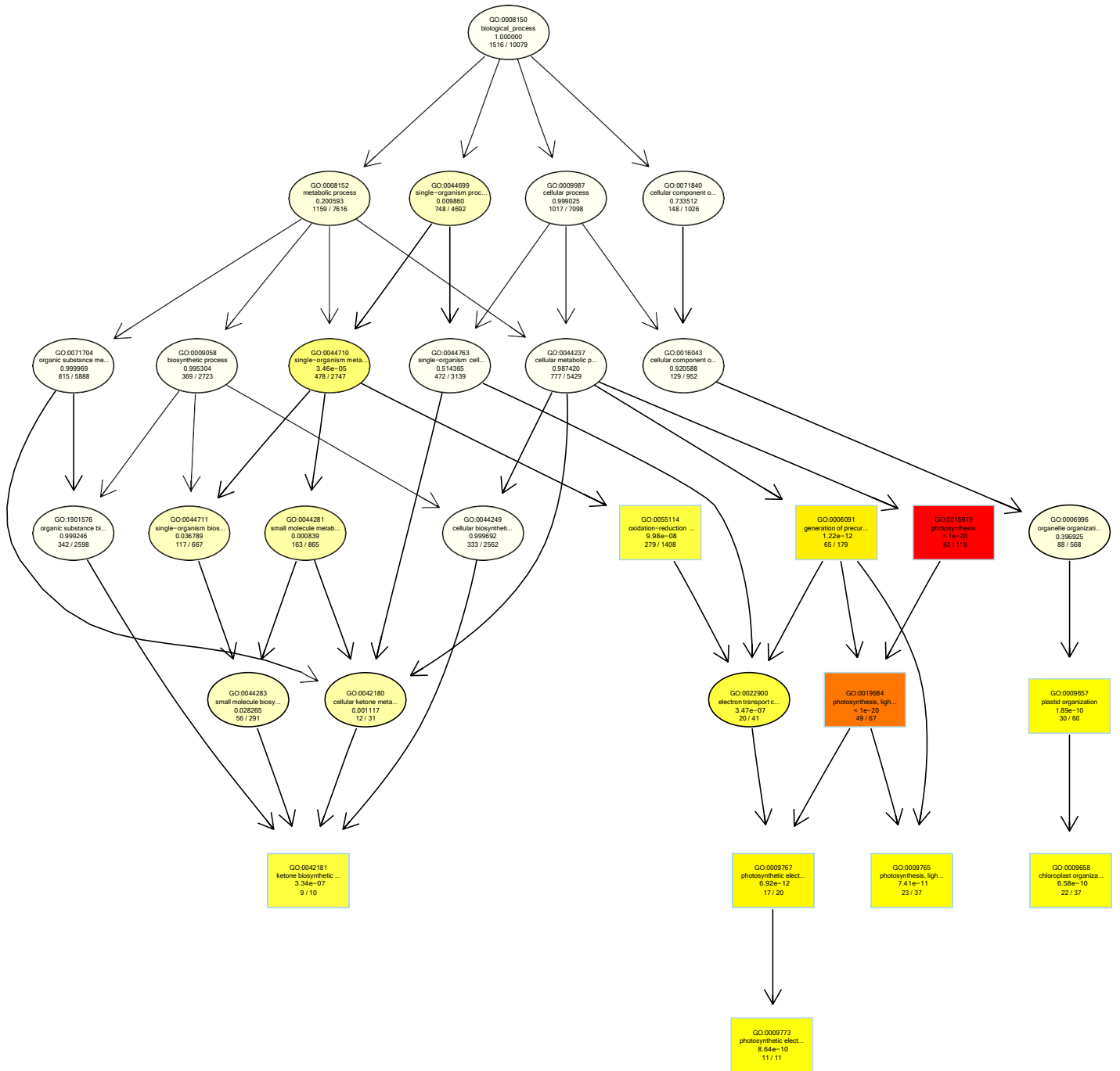


Figure S10: Biological processes that DEGs between etiolated and green wheat seedlings on vernalization 40 d involved in, revealed by topGO enrichment analysis.

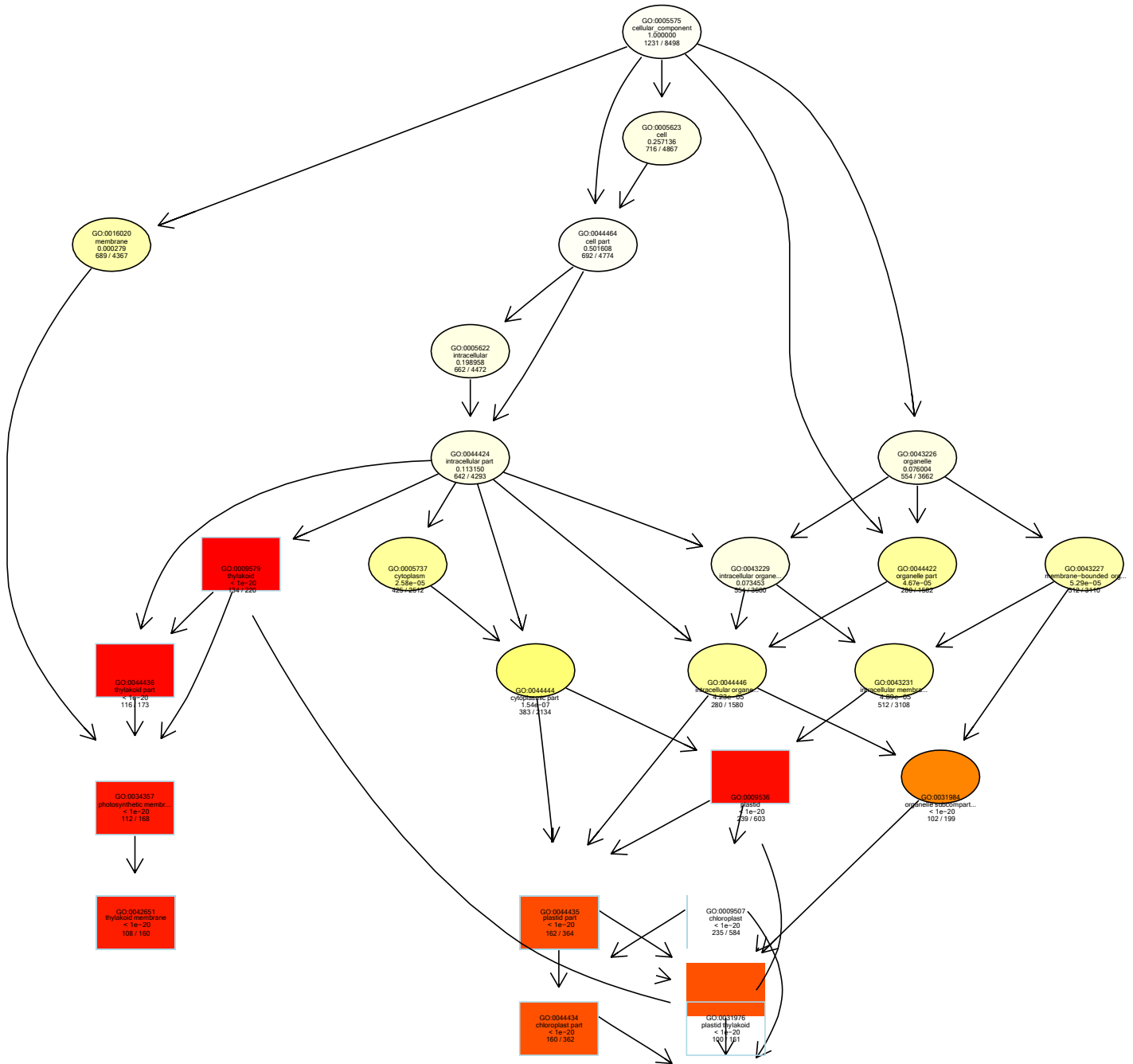


Figure S11: Cellular component where DEGs were found, revealed by topGO enrichment analysis. The DEGs were between etiolated and green wheat seedlings on vernalization 40 d.

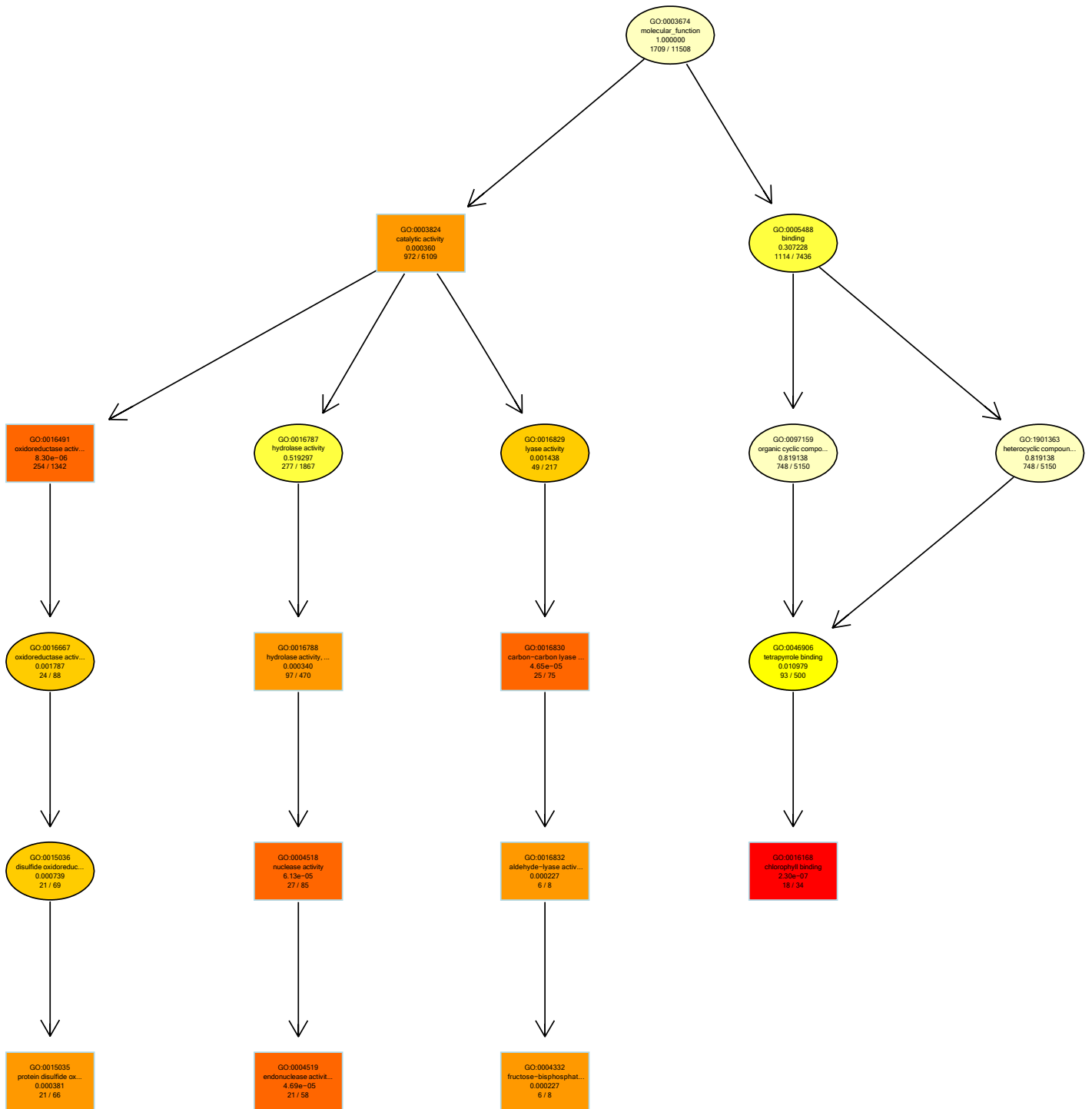


Figure S12: Molecular functions of DEGs between etiolated and green wheat seedlings on vernalization 40 d, revealed by topGO enrichment analysis.