Microarray analysis of E2Fa-DPa-overexpressing plants uncovers a cross-talking genetic network between DNA replication and nitrogen assimilation

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Summary
Previously we have shown that overexpression of the heterodimeric E2Fa-DPa transcription factor in Arabidopsis thaliana results in ectopic cell division, increased endoreduplication, and an early arrest in development. To gain a better insight into the phenotypic behavior of E2Fa-DPa transgenic plants and to identify E2Fa-DPa target genes, a transcriptomic microarray analysis was performed. Out of 4,390 unique genes, a total of 188 had a twofold or more up- (84) or down-regulated (104) expression level in E2Fa-DPa transgenic plants compared to wild-type lines. Detailed promoter analysis allowed the identification of novel E2Fa-DPa target genes, mainly involved in DNA replication. Secondarily induced genes encoded proteins involved in cell wall biosynthesis, transcription and signal transduction or had an unknown function. A large number of metabolic genes were modified as well, among which, surprisingly, many genes were involved in nitrate assimilation. Our data suggest that the growth arrest observed upon E2Fa-DPa overexpression results at least partly from a nitrogen drain to the nucleotide synthesis pathway, causing decreased synthesis of other nitrogen compounds, such as amino acids and storage proteins.

Key words: Arabidopsis thaliana, Cell cycle, E2F, Microarray, Nitrogen assimilation

Introduction
Progression through the cell cycle is essential for the continued existence of all uni- and multicellular organisms. It is crucial for the survival of a cell that its DNA is correctly replicated. In mammals, the onset of DNA replication is regulated by the activity of the heterodimeric E2F-DP transcription factor. The mammalian E2F family contains six proteins (E2F1, E2F2, E2F3, E2F4, E2F5 and E2F6) (Trimarchi and Lees, 2002). All E2Fs have an N-terminally located DNA-binding domain immediately followed by a dimerization domain, allowing them to pair with a dimerization partner (DP1 or DP2). Dimerization of E2F with DP is a prerequisite for high affinity, sequence-specific binding to the E2F consensus DNA-binding site. E2F activity is negatively regulated by retinoblastoma (Rb), which binds to the transcriptional activation domain of the E2F-DP factor, rendering it inactive. Moreover, the recruitment by Rb of DNA-modifying enzymes, such as histone deacetylases and polycomb proteins, leads to chromatin condensation with suppression of promoter activity of E2F-DP target genes as a result. Phosphorylation of Rb by cyclin-dependent kinases (CDKs) counteracts its inhibitory function, resulting in the release of transcriptionally active E2F-DP and consequential onset of DNA replication.

The mechanism of DNA replication seems to be conserved between mammals and plants, because E2F and DP genes have been isolated from different plant species, including wheat, tobacco, carrot, Arabidopsis and rice (Ramírez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000; Ramírez-Parra and Gutierrez, 2000; Kosugi and Ohashi, 2002a). In the Arabidopsis genome there are three E2F (E2Fa, E2Fb and E2Fc) and two DP (DPa and DPa) genes (Vandepoele et al., 2002). Recently, we have analyzed the phenotypes of plants co-overexpressing the E2Fa-DPa genes (De Veylder et al., 2002). Transgenic plants were smaller than control plants, had curled leaves and cotyledons, and were arrested in growth at an early stage of development. Microscopic analysis revealed that E2Fa-DPa-overproducing cells underwent ectopic cell division or endoreduplication, depending on the cell type. Whereas extra cell divisions resulted in cells smaller than those seen in the same tissues of control plants, supplementary endoreduplication caused the formation of giant nuclei. By using reverse transcription (RT)-PCR, we demonstrated that the expression levels of genes involved in DNA replication (CDC6, ORC1, MCM and DNA pol α) were strongly up-regulated (De Veylder et al., 2002).

Physiologically important targets of the mammalian E2F-DP transcription factors have been identified by microarray hybridization experiments, chromatin immunoprecipitations and computer-assisted prediction (Ishida et al., 2001; Kel et al., 2001; Müller et al., 2001; Weinmann et al., 2001; Ren et al., 2002). E2F-DP-responsive genes can be found among genes involved in cell division, DNA repair and replication, mitotic
progression, apoptosis and differentiation. Although little is known about the plant E2F-DP target genes, a database search has been published recently, in which the Arabidopsis genome was screened for genes harboring the TTCCCCGCC cis-acting element in their promoter (Ramirez-Parra et al., 2003). However, it is still unclear whether this specific cis-acting element is the only one recognized by the plant E2F-DP complex, or whether the presence of the TTCCCCGCC element is sufficient to mark a gene as a true E2F-DP target gene. In order to identify the functional classes of genes regulated by E2Fa-DPa and to understand the nature of the phenotype of the E2Fa-DPa-overexpressing plants, we designed a microarray experiment that compared the transcript levels of 4,571 genes of wild-type and transgenic lines. We found distinct classes of genes that were up- or down-regulated in the E2Fa-DPa plants. Promoter analysis allowed us to distinguish among the downstream expressed genes, the genes that were putatively under direct control of E2Fa-DPa. Furthermore, we found that the increased expression levels of E2Fa-DPa have a large impact on the expression levels of genes involved in nitrogen assimilation and metabolism.

Materials and Methods

Plant material

Double transgenic CmMV35S-E2Fa-DPa plants were obtained by crossing homozygous CmMV35S-E2Fa and CmMV35S-DPa plants (De Veylder et al., 2002). Double transformants were grown under a 16-hour light/8-hour dark photoperiod at 22°C on germination medium (Valvekens et al., 1988).

Construction of microarrays

The Arabidopsis thaliana (L.) Heynh. microarray consisted of 4,608 cDNA fragments spotted in duplicate, distant from each other, on Type V silane-coated slides (Amersham Biosciences, Little Chalfont, UK). The clone set included 4,571 Arabidopsis cDNAs from the unigene clone collection Arabidopsis Gem I (Incyte Genomics, Palo Alto, CA). The functional annotation of the genes related to the spotted cDNAs was retrieved by BLASTN against genomic sequences. To facilitate the analysis, a collection of genomic sequences was built each bearing only one gene. In each of these sequences, the upstream intergenic sequence was followed by the exon-intron structure of the gene and the downstream intergenic sequence, or, in other words, the whole genomic sequence between start and stop codons from neighboring protein-encoding genes. From the BLASTN output, the best hits were extracted and submitted to a BLASTX search against protein databases. From this analysis, the set of 4,571 cDNAs appeared to constitute 4,390 unique clones. To obtain more detailed information concerning the potential function of the genes, protein domains were searched using ProDom. The complete set can be found at http://www.psb.ugent.be/E2F/. The cDNA inserts were amplified by PCR with M13 primers, purified with MultiScreen-PCR plate (Millipore, Bedford, MA), and arrayed on slides using a Generation III printer (Amersham Biosciences). Slides were blocked in 3.5% SSC (1× SSC) 100 mM NaCl, 15 mM sodium citrate, pH 7.0, 0.2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin for 10 minutes at 60°C.

RNA amplification and labeling

Antisense RNA was amplified with a modified protocol of in vitro transcription (Puskás et al., 2002). For the first-strand cDNA synthesis, 5 μg of total RNA was mixed with 2 μg of a HPLC-purified anchored oligo(dT) + T7 promoter (5'-GGCCAGTGAATTTGTAATAGCAGCT-CACTATGGAGGGCGG-T3) (Eurogentec, Seraing, Belgium), 40 units of RNaseOUT (Invitrogen, Gaithersburg, MD) and 0.9 M D(+)-trehalose (Sigma-Aldrich, St. Louis, MO) in a total volume of 11 μl and heated to 75°C for 5 minutes. To this mixture, 4 μl 5X first-strand buffer (Invitrogen), 2 μl 0.1 M dithiothreitol, 1 μl 10 mM dNTP mix, 1 μl 1.7 M D(+)-trehalose (Sigma-Aldrich), and 1 μl SuperScript II (Invitrogen) were added to 20 μl final volume. The sample was incubated in a UnOII thermocycler (Whatman Biometra, Göttingen, Germany) at 37°C for 5 minutes, at 45°C for 10 minutes, 10 cycles at 60°C for 2 minutes and at 55°C for 5 minutes. To the first-strand reaction mix, 103.8 μl water, 33.4 μl 5X second-strand synthesis buffer (Invitrogen), 3.4 μl 10 mM dNTP mix, 1 μl of 10 U/μl DNA ligase (Invitrogen), 4 μl 10 U/μl DNA Polymerase I (Invitrogen), and 1 μl 2 U/μl RNase H (Invitrogen) were added and incubated at 16°C for 2 hours. The synthesized double-stranded cDNA was purified with Qiapquick (Qiagen, Hilden, Germany). Antisense RNA was synthesized by AmpliScribe T7 high-yield transcription kit (Epiconcent Technologies, Madison, WI) in a total volume of 20 μl according to the manufacturer’s instructions. The RNA was purified with the RNeasy purification kit (Qiagen). From this RNA, 5 μg was labeled by reverse transcription using random nonamer primers (Genset, Paris, France), 0.1 mM d/d(T/A)TPs, 0.05 mM dCTP (Amersham Biosciences), 0.05 mM Cy3-dCTP or Cy5-dCTP (Amersham Biosciences), 1× first-strand buffer, 10 mM dithiothreitol, and 200 U of SuperScript II (Invitrogen) in 20 μl total volume. The RNA and primers were denatured at 75°C for 5 minutes and cooled on ice before the remaining reaction components were added. After 2 hours incubation at 42°C, mRNA was hydrolyzed in 250 mM NaOH for 15 minutes at 37°C. The sample was neutralized with 10 μl of 2 M 3-(N-morpholino)propanesulfonic acid and purified with Qiapquick (Qiagen).

Array hybridization and post-hybridization processes

The probes were resuspended in 30 μl hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA) and prehybridized with 1 μl poly(dT) (1 mg/ml) at 42°C for 30 minutes to block hybridization on the polyA/T tails of the cDNA on the arrays. Mouse COT DNA (1 mg/ml) (Invitrogen) was added to the mixture and placed on the array under a glass coverslip. Slides were incubated for 18 hours at 42°C in a humid hybridization cabinet (Amersham Biosciences). Post-hybridization washing was performed for 10 minutes at 56°C in 1× SSC, 0.1% SDS, twice for 10 minutes at 56°C in 0.1× SSC, 0.1% SDS, and for 2 minutes at 37°C in 0.1× SSC.

Scanning and data analysis

Arrays were scanned at 532 nm and 635 nm using a Generation III scanner (Amersham Biosciences). Image analysis was performed with ArrayVision (Imaging Research Inc, St. Catharines, Ontario, Canada). Spot intensities were measured as artifact-removed total intensities (ARV ol) without correction for background. We first addressed within-slide normalization by plotting for each single slide a ‘MA-plot’ (Yang et al., 2002), where M=log2(R/G) and A=log2(R×G). Dye intensity differences were corrected with the ‘LOWESS’ normalization. Subsequently, between-slide normalization and differentially expressed gene identification between the two genotypes were performed by sequential analysis of variances (ANOVAs), according to the method of Wollinger et al. (Wollinger et al., 2001). (i) The base-2 logarithm of the ‘LOWESS’-transformed measurements for all 73,136 spots (ykilm) were subject to a normalization model ykilm = μ + A + Dijkl + eijklm, where μ is the sample mean, A the effect of the kth array (k = 1–4), Dijkl the channel effect (AD) for the mth replication (m = 2; left and right) of the total collection of l = 1, ..., 4571 cDNA fragments, and eijklm the stochastic error. (ii) The residuals from this model were subjected to 4,571 gene-specific models rijklm = G(c)A + c(i)D + G(c)+fjg, where G(c) is the spot effect, G(c)+ the gene-specific dye effect, G(c) the signal intensity for genes that can
specifically be attributed to the genotypes (effect of interest), and \( \gamma_{ijk} \) the stochastic error. All effects were assumed to be fixed, except for \( \varepsilon_{ik} \) and \( \gamma_{ijk} \). T-tests for differences between the GC effects were performed, all based on \( n_1+n_2-6 \) degrees of freedom, where \( n_1 \) and \( n_2 \) correspond to the number of wild-type and E2Fa-DPa hybrids, respectively. Bonferroni adjustment for the 4,571 tests assures an experiment-wise false positive rate of 0.05 results in a \( P \)-value cut-off of 1e-5.0, which is certainly too conservative. Thus, no further adjustments for multiple testing were done. Therefore, we chose to set the \( P \)-value cut-off arbitrarily at the 0.05 level. We used Genstat for both the normalization and gene model fits.

RT-mediated PCR analysis

RNA was isolated from plants 8 days after sowing with the Trizol reagent (Amersham Biosciences). First-strand cDNAs were prepared from 3 \( \mu \)g of total RNA with the Superscript RT II kit (Invitrogen) and oligo(dT)15 according to the manufacturer’s instructions. A 0.25 \( \mu \)l aliquot of the total RT reaction volume (20 \( \mu \)l) was used as a template in a semi-quantitative RT-mediated PCR amplification, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. From the PCR reaction, 10 \( \mu \)l was separated on a 0.8% agarose gel and transferred onto Hybond N+ membranes (Amersham Biosciences) that were hybridized at 65\( ^\circ \)C with fluorescein-labeled probes (Genes Images random prime probe; Amersham Biosciences). The hybridized bands were detected with the CDP Star detection module (Amersham Biosciences). Primers used were 5'-AAAAGCAGGCCTTGTCGACGATCTCCTTCCCGG-3' and 5'-AGAAAGCCTGCTCATGTAGAGGAACCGC-3' for E2Fa, 5'-ATAGAATTCGCTTACATTTCATTTGGAAACTGATG-3' and 5'-ATAGTCGACTCAGCGAGTATCAATGGATCC-3' for \( \Delta HAT B \) and 5'-GTATTAGCTGCTATGAGGCA-3' for glutamine synthetase (GS), 5'-GGTCAAAAA-GATAACACACACCG-3' for glutamate synthase (GOGAT), 5'-CGTCTTCCTGAC-3' and 5'-GAACGCCATTGAGAAAGTCTGAC-3' for histone acetylase \( HAT B \), 5'-GGTATTAGCTGCTATGAGGCA-3' for glutamine synthetase (GOGAT), 5'-CGTCTTCCTGAC-3' and 5'-GAACGCCATTGAGAAAGTCTGAC-3' for histone acetylase \( HAT B \), 5'-GTATCCGGCTGACT-TGAAAGTAC-3' and 5'-GAATCCGGAGGGAAAGTCTGAC-3' for LOB domain protein 41, 5'-GTGTGTTGTTTCAAAGTTTCCCTACG-3' and 5'-GGTAAAGGACTAGGCTTGTG-3' for isocitrate lyase, 5'-GGGTCAAAAGATAACACACACCG-3' and 5'-CCGCCCTTAT-TATTAGCCGAC-3' for nitrite reductase (NiR), and 5'-TTACC-GAGGCTCCTTCAACC-3' and 5'-ACCACCGATCCGACACT-GTAC-3' for actin 2 (ACT2).

Promoter analysis

The intergenic sequence corresponding to the promoter area of each gene spotted on the microarray was deduced from genomic sequences. From these intergenic sequences, up to 500 bp upstream of the ATG start codon were extracted and subjected to motif searches to retrieve potential E2F elements. Of the 4,571 expressed sequence tags (ESTs) spotted on the microarray, we could retrieve the genomic sequence of 4,390. This difference is due to the presence of duplicate genes and mitochondrial or chloroplast DNA on the microarray. The position and frequency of occurrence were determined with the publicly available MatInspector (version 2.2) by using matrices extracted from PlantCARE and matrices made especially for this particular analysis (Lescot et al., 2002). The relevance of each motif was evaluated against a background consisting of all the sequences from the dataset using the Fisher exact test.

Results and Discussion

Experimental setup and statistical analysis

A microarray containing in replicate 4,571 Arabidopsis expressed sequence tags (ESTs) was used to compare the transcriptome of the wild-type with that of E2Fa-DPa-overexpressing plants. cDNA was synthesized from total RNA of plants harvested 8 days after sowing. At that stage, transgenic plants can be distinguished from control plants by the appearance of curled cotyledons that display ectopic cell divisions and enhanced endoreduplication (De Veylder et al., 2002). In the first two hybridizations, including a biological repeat, fluorescently Cy3- and Cy5-labeled probe pairs of control and E2Fa-DPa cDNAs were used. Subsequently, a dye-swap replication was performed for both hybridizations, resulting in a total of four cDNA microarray hybridizations. Because each cDNA was printed in duplicate on the array, eight data points for every gene were obtained.

Fluorescence levels were analyzed to establish whether the expression level of each gene varied according to the overexpression of the E2Fa-DPa transcription factor. Two sequential ANOVA models were used, as proposed by Wolflinger et al. (Wolflinger et al., 2001). First, the model called ‘normalization model’ accounts for experiment-wise systematic effects, such as array and channel effects, which could bias inferences made on the data from the individual genes. The residuals from this model represent normalized values and are the input data for the second model, called the ‘gene’ model. The gene models are fitted separately to the normalized data from each gene (see Materials and Methods). In this procedure, normalized expression levels rather than ratios are used as units.

For each of the 4,571 genes on the arrays the genotypetype-specific signal intensity was determined and \( t \)-tested for significant differences \((P<0.05)\). Fig. 1 presents the \( P \) values obtained (as the negative log10 of the \( P \) value) against the
### Table 1. *Arabidopsis* genes up-regulated twofold or more in E2Fa-DPa plants sorted according to functional category

| Gene identification | Accession number | ORF name | Fold induction | E2F motif | Position* | Strand |
|---------------------|------------------|----------|----------------|-----------|-----------|--------|
| **DNA replication and modification (14)** | | | | | | |
| Putative thymidine kinase | AI997851 | At3g07800 | 8.44 | | | |
| DNA methyltransferase | AI994691 | At5g49160 | 5.37 | | | |
| Ms3 | AW004204 | At4g35050 | 4.89 | TTTCGCCG | -75 | |
| Putative linker histone protein | AI994590 | At3g18035 | 3.31 | | | |
| Putative replication factor c | AI997934 | At1g21690 | 3.30 | TTTCGCCG | -96 | |
| Topoisomerase 6 subunit A | AI995290 | At5g02820 | 2.62 | TTTCGCCG | -66 | +
| **Histone H4-like protein** | AI999171 | At3g46320 | 2.55 | TTTCGCCG | -310 | +
| **Histone acetylase HAT B** | AI998229 | At5g06740 | 2.36 | TTTCGCCG | -50 | +
| **Putative DNA gyrase subunit A** | AI995400 | At5g02820 | 2.62 | TTTCGCCG | -66 | +
| **Histone H2A-like protein** | AI995882 | At4g27230 | 2.23 | | | |
| **Putative mismatch binding protein** | AI993280 | At3g24320 | 2.10 | | | |
| **Adenosyl homocysteinase** | AI996953 | At4g13940 | 2.07 | | | |
| **Cell cycle (2)** | | | | | | |
| **E2Fa** | AI294534 | At2g36010 | 94.88 | | | |
| **CDK1:1** | D10851 | At3g54180 | 2.60 | TTTCGCCG | -151 | -
| **Cell wall biogenesis (11)** | | | | | | |
| **Xyloglucan endo-1,4-β-D-glucanase (meri-5)** | AI994459 | At4g30270 | 3.74 | | | |
| Putative glycosyltransferase | AI999244 | At1g70090 | 3.38 | | | |
| α-Galactosyltransferase-like protein | AI998223 | At3g06220 | 2.32 | | | |
| Putative xyloglucan endotransglycosylase | AI999683 | At4g32730 | 2.85 | | | |
| Xyloglucan endo-1,4-β-D-glucanase-like protein | AI998301 | At4g30280 | 2.74 | TTTCGCCG | -66 | +
| Putative xyloglucan endotransglycosylase | AI994477 | At1g14720 | 2.51 | TTTCGCCG | -96 | |
| Putative glycosyltransferase | AI999770 | At1g24170 | 2.39 | TTTCGCCG | -310 | -
| Putative UDP-glucose glucosyltransferase | AI997288 | At1g22400 | 2.34 | | | |
| **Putative glucosyltransferase** | AI998872 | At2g15480 | 2.15 | | | |
| Peroxidase | AI994622 | At2g38380 | 2.11 | TTTCGCCG | -314 | -
| β-1,3-glucanase-like protein | AI994681 | At3g55430 | 2.05 | | | |
| **Chloroplastic genes (7)** | N96785 | rbcL | 4.71 | | | |
| Large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase RbcL | AI994194 | rpl33 | 3.54 | TTTCGCCG | -315 | -
| Photosystem II protein | AW004203 | PsbI | 2.81 | | | |
| Ribosomal protein L2 | AW004266 | rpl2 | 2.61 | TTTCGCCG | -315 | -
| **ATP-dependent protease subunit** | AI997947 | At1g24170 | 2.39 | | | |
| **Cytochrome B6** | AI997102 | PetB | 2.55 | TTTCGCCG | -36 | -
| **ATPase 8 subunit** | AI994251 | atpE | 2.17 | TTTCGCCG | -160 | -
| **Mitochondrial genes (1)** | | | | | | |
| 26S ribosomal RNA protein | AW004275 | orf107a | 2.87 | | | |
| **Transcription factors (6)** | | | | | | |
| LOB domain protein 41 | AI996685 | At3g02550 | 4.01 | TTTCGCCG | -23 | -
| WRKY transcription factor 21 | AI992739 | At2g30890 | 2.78 | TTTCGCCG | -23 | -
| GATA Zn-finger protein | AI995736 | At3g16870 | 2.75 | | | |
| Anthocyaninless2 | AI993655 | At4g00730 | 2.73 | | | |
| Leucine zipper-containing protein | AI995691 | At1g07000 | 2.43 | TTTCGCCG | -33 | +
| Homeodomain transcription factor (Athb-6) | AI999190 | At2g22430 | 2.30 | | | |
| **Metabolism and biogenesis (11)** | | | | | | |
| Alcohol dehydrogenase | AI998773 | At1g77120 | 5.09 | | | |
| Putative isocitrate lyase | AI999168 | At3g21720 | 3.08 | | | |
| Protoporphyrilide reductase precursor | AI993342 | At2g17640 | 2.39 | | | |
| Sugar transporter-like protein | AI997793 | At4g36670 | 2.27 | | | |
| NADH-dependent glutamate synthase (GOGAT) | AI997600 | At5g53460 | 2.25 | TTTCGCCG | -225 | +
| Nitrate reductase (NIA2) | AI996208 | At1g37130 | 2.15 | | | |
| Pectate lyase-like protein | AI980995 | At4g54920 | 2.13 | TTTCGCCG | -225 | +
| Putative sterol dehydrogenase | AI996340 | At1g43420 | 2.10 | TTTCGCCG | -225 | +
| Glutamine synthetase root isozyme 1 (GS) | AI996200 | At1g66200 | 2.06 | | | |
| Monosaccharide transporter STP3 | AI997045 | At5g61520 | 2.05 | | | |
| **Signal transduction (6)** | | | | | | |
| Calcium-dependent protein kinase | AI996555 | At5g66210 | 2.96 | | | |
| WD-40 repeat protein | AI993055 | At5g14530 | 2.70 | TTTCGCCG | -104 | -
| Receptor-protein kinase-like protein | AI994727 | At5g53480 | 2.59 | | | |
| Putative phytochrome A | AI998146 | At1g09570 | 2.45 | | | |
| Putative leucine-rich receptor-like protein kinase | AI996961 | At1g72180 | 2.13 | | | |
| Putative receptor-like kinase | AI993298 | At2g23750 | 2.06 | | | |
magnitude of the effect (log2 of estimated fold change). This so-called volcano plot illustrates the substantial difference of significance testing as opposed to cut-offs strictly based on the fold change. The two vertical reference lines indicate a twofold cut-off for either repression or induction, whereas the horizontal reference line refers to the P-value cut-off at 0.05. These reference lines divide the plot into six meaningful sectors. The 3,126 genes in the lower middle sector have low significance and low fold change, and both methods are in agreement that the corresponding changes are not significant. The 188 genes in the upper left and right sectors have high significance (P < 0.05) and high fold change (≥ 2); 84 of these genes show a significant two-or-more-fold induction of expression, whereas the remaining 104 genes show a significant two-or-more-fold repression of expression in the E2Fa-DPa plants. The identity of these genes was confirmed by sequencing, and the induction of a random set of selected genes was confirmed by RT-PCR analysis (Fig. 2). Finally, the 1,257 genes in the upper middle sector represent significant (P < 0.05) up- or down-regulated genes, but with a low (≤ 2) fold change. The full dataset of genes can be viewed at http://www.psb.ugent.be/E2F/.

DNA replication and cell cycle genes

Genes up- or down-regulated in the E2Fa-DPa transgenic plants can be classified into clear groups according to their function (Tables 1 and 2). Among the genes that are twofold or more up-regulated, 14 belong to the class of DNA replication and modification, correlating with the observation that E2Fa-DPa-overexpressing plants undergo extensive endoreduplication. Most of these genes have previously been
### Table 2. Arabidopsis genes repressed twofold or more in E2Fa-DPa plants sorted according to functional category

| Gene identification | Accession number | ORF name | Fold repression | E2F motif | Position* Strand |
|---------------------|------------------|----------|----------------|-----------|-----------------|
| **Cell wall biogenesis** (4) | | | | | |
| Similar to polygalacturonase-like protein | AI993509 | At1g10640 | 3.62 | | |
| Putative xyloglucan endo-transglycosylase | AI997647 | At2g36870 | 2.51 | | |
| Pectate lyase 1-like protein | AI994801 | At1g67750 | 2.40 | | |
| Xyloglucan endo-transglycosylase | AI98832 | At3g44990 | 2.35 | | |
| **Metabolism and biogenesis** (24) | | | | | |
| Fructose-bisphosphate aldolase-like protein | AI994456 | At4g26530 | 5.99 | ATTGGCCC | –426 – |
| Sucrose-phosphate synthase-like protein | AI995432 | At4g10120 | 4.64 | | |
| Putative β-fructosidase | AI994670 | At1g62660 | 2.66 | TTTCCCCC | –344 – |
| Neoxanthin cleavage enzyme-like protein | AI997269 | At4g19170 | 2.66 | | |
| Putative β-amylase-like protein | AI999322 | At5g18670 | 2.53 | | |
| Cytochrome P450 monoxygenase (CYP83A1) | AI994017 | At4g13770 | 2.57 | | |
| FRO1-like protein; NADPH oxidase-like | AI995306 | At4g27820 | 2.20 | ATTGGCCC | –327 – |
| Putative glutathione peroxidase | AW004143 | At2g25080 | 2.15 | | |
| Putative adenosine phosphosulfate kinase | AW004219 | At2g14750 | 2.13 | | |
| Tyrosine transaminase-like protein | AI96914 | At4g23600 | 2.13 | | |
| **Transcription factors** (5) | | | | | |
| Homeobox-leucine zipper protein ATHB-12 | AI994027 | At3g61890 | 4.20 | ATTGGCCG | –113 – |
| NAC domain protein NAC2 | AI992865 | At1g69490 | 3.68 | | |
| MYB-related transcription factor | AI995298 | At1g71030 | 2.78 | | |
| Dof zinc finger protein | AI994875 | At1g51700 | 2.30 | TTTCGCCG | –18 + |
| MYB-related transcription factor (CCA1) | AI992931 | At2g46830 | 2.19 | TTTCGCCG | –13 – |
| **Signal transduction** (9) | | | | | |
| Serine/threonine protein kinase-like protein | AI995557 | At5g10930 | 3.91 | | |
| Putative β-glucosidase | AI993428 | At4g21650 | 3.19 | TTTCGCCG | –85 + |
| Putative lectin | AI996160 | At4g10770 | 2.68 | | |
| Putative lectin | AI998542 | At3g16400 | 2.52 | | |
| Ca2+-dependent membrane-binding protein annexin | AI998553 | At1g35720 | 2.45 | | |
| Putative WD repeat protein | AI997328 | At3g15880 | 2.38 | | |
| Putative lectin | AI999016 | At3g16390 | 2.35 | | |
| Putative lectin | AI993358 | At3g16530 | 2.31 | | |
| **Others** (25) | | | | | |
| Putative protease inhibitor Dr4 | AI995265 | At1g73330 | 10.30 | | |
| Major latex protein homolog-like | AI998305 | At2g01520 | 4.27 | | |
| Pollen allergen-like protein | AI993041 | At1g24020 | 3.56 | TTTCGCCG | –377 + |
| Putative heat shock protein | AI997846 | At1g06460 | 3.55 | | |
| Putative fibrillin | AI997199 | At4g04020 | 3.55 | TTTCGCCG | –435 – |
| Major latex protein homolog-like | AI997255 | At1g70890 | 3.50 | | |
| Putative nematode resistance protein | AI993740 | At2g40000 | 2.95 | | |
| Putative auxin-regulated protein | AI996069 | At2g46690 | 2.86 | | |
| Putative myrosinase-binding protein | AI997583 | At2g39310 | 2.61 | | |
| Ubiquitin-conjugating enzyme-like protein | AI997782 | At5g56150 | 2.41 | | |
| Ubiquitin-conjugating enzyme E2–KDa 8 | AI994771 | At5g41700 | 2.40 | | |
| Vegetative storage protein Vsp2 | AI999152 | At5g24770 | 2.35 | | |
| Heat shock protein 70 | AI994044 | At3g12580 | 2.24 | | |
| Chloroplast outer envelope membrane protein | AI997015 | At3g63160 | 2.20 | | |
| Translation initiation factor-like protein | AI992786 | At5g54900 | 2.15 | | |
| Pseudogene | AI995323 | At2g04110 | 2.07 | | |
| Vegetative storage protein Vsp1 | AI999546 | At5g24780 | 2.06 | | |
| Dehydrin ERD10 | AI997518 | At1g20450 | 2.06 | | |
| MTN3-like protein | AI997159 | At3g48740 | 2.05 | | |
| Putative chlorophyll a/b-binding protein | AI994859 | At3g27690 | 2.05 | | |
| Photosystem I reaction center subunit psaN | AI997939 | At5g64040 | 2.03 | | |
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shown to be up-regulated by E2F-DP overexpression in mammalian cells, including a putative thymidine kinase, replication factor c, adenosylhomocysteinase, DNA (cytosine-5)-methyltransferase, and histone genes (Ishida et al., 2001; Müller et al., 2001; Ren et al., 2002). Other E2Fa-DPa-induced S phase genes include a linker histone protein, the topoisomerase 6 subunit A, and two subunits of the histone acetyltransferase HAT B complex, namely HAT B and Msi3. The HAT B complex is responsible for the specific diacetylation of newly synthesized histone H4 during nucleosome assembly on newly synthesized DNA (Lusser et al., 1999).

In addition to the overexpressed E2Fa gene (90-fold more abundant in transgenic than in control plants), only one cell cycle gene (CDKB1;1) has a twofold or more change in expression level upon E2Fa-DPa overexpression. CDKB1;1 had already been predicted to be a candidate E2F-DP target by the presence of a consensus E2F-DP-binding site in its promoter (de Jager et al., 2001). Whereas CDKB1;1 activity is highest at the G2 to M transition, its transcript levels start to increase during S phase (Porceddu et al., 1999; Menges and Murray, 2002). Therefore, up-regulation of CDKB1;1 might be a mechanism linking DNA replication with the following mitosis. That other cell cycle genes modulated in the E2Fa-DPa plants are not detected can be explained by the lack of many important E2F-DP target genes on the microarray and the putative difficulty in identifying changes in expression levels of lowly expressed genes in microarray hybridizations.

Cell wall biogenesis genes

Four members of the xylol glucan endotransglucosylase (XET) gene family are found to be twofold or more up-regulated in the E2Fa-DPa plants, one of them identical to the previously

Table 2. Continued

| Gene identification | Accession number | ORF name | Fold repression | E2F motif | Position* | Strand |
|---------------------|-----------------|---------|----------------|-----------|-----------|--------|
| Others – continued  |                 |         |                |           |           |        |
| AR781 similar to yeast pheromone receptor | AI998194 | At2g26530 | 2.03 |            |           |        |
| Putative lipid transfer protein | AI997024 | At2g13050 | 2.03 |            |           |        |
| Peroxidase ATP3a | AI998372 | At5g61400 | 2.03 | TTTGGCGG | –492 | + |
| Myosin heavy chain-like protein | AI999224 | At3g16000 | 2.01 |            |           |        |
| Unknown function (35) |                 |         |                |           |           |        |
| Unknown protein | AI993767 | At1g45200 | 3.91 |            |           |        |
| Putative protein | AI993468 | At3g56290 | 3.38 |            |           |        |
| Hypothetical protein | AI996374 | At1g61890 | 2.78 |            |           |        |
| Unknown protein | AI994573 | At3g15950 | 2.71 |            |           |        |
| Putative protein | AI994726 | At3g52360 | 2.65 |            |           |        |
| Hypothetical protein | AI997393 | At4g02920 | 2.60 | TTTGCCCC | –419 | – |
| Unknown protein | AI508997 | At5g43580 | 2.58 |            |           |        |
| Unknown protein | AI997866 | At1g70760 | 2.52 |            |           |        |
| Unknown protein | AI997085 | At5g43750 | 2.51 |            |           |        |
| Putative protein | AI995724 | At5g50100 | 2.48 |            |           |        |
| Unknown protein | AI995337 | At1g74880 | 2.42 |            |           |        |
| Unknown protein | AI998296 | At3g19370 | 2.40 |            |           |        |
| Unknown protein | AI993346 | At3g10420 | 2.40 |            |           |        |
| Putative protein | AI999485 | At3g61080 | 2.38 |            |           |        |
| Unknown protein | AI996923 | At1g67860 | 2.38 |            |           |        |
| Unknown protein | AI994841 | At1g52870 | 2.35 | ATTCCCC | –74 | + |
| Unknown protein | AI999581 | At1g64370 | 2.35 |            |           |        |
| Unknown protein | AI997584 | At1g05870 | 2.25 |            |           |        |
| Putative protein | AI992938 | At5g03540 | 2.21 |            |           |        |
| Hypothetical protein | AI997712 | At2g15020 | 2.21 |            |           |        |
| Unknown protein | AI998338 | At1g68440 | 2.20 |            |           |        |
| Unknown protein | AI996872 | At2g21960 | 2.19 |            |           |        |
| Putative protein | AI996295 | At4g27280 | 2.18 |            |           |        |
| Putative protein | AI995642 | At3g48200 | 2.16 |            |           |        |
| Unknown protein | AI997470 | At2g32870 | 2.14 |            |           |        |
| Hypothetical protein | AI998460 | At1g69510 | 2.11 | ATTCGGG | –120 | + |
| Putative protein | AI993356 | At5g22460 | 2.10 | TTTGGCC | –492 | + |
| Putative protein | AI995956 | At5g20650 | 2.08 |            |           |        |
| Unknown protein | AI996100 | At2g35830 | 2.06 |            |           |        |
| Hypothetical protein | AI996039 | At3g27050 | 2.05 | ATTCGCC | –5 | – |
| Unknown protein | AI996020 | At5g17200 | 2.04 |            |           |        |
| Putative protein | AI996010 | At4g39730 | 2.03 |            |           |        |
| Hypothetical protein | AI998372 | At2g01260 | 2.03 |            |           |        |
| Unknown protein | AI999573 | At3g61060 | 2.00 |            |           |        |
| Unknown protein | AI998562 | At2g35760 | 2.00 |            |           |        |
| No hit (2) |                 |         |                |           |           |        |
| No hit on genome | AI995690 | 2.54 |            |           |           |        |
| No hit on genome | AI990010 | 2.23 |            |           |           |        |

*Relative position upstream from the translation initiation site.
described Meri-5 gene (Medford et al., 1991). XETs are enzymes that modify cell wall components and are presumed to play a role in altering size, shape and physical properties of plant cells. Reversal breakage of the xyloglucan tethers by XETs has been proposed as a mechanism for allowing cell wall loosening in turgor-driven cell expansion (Campbell and Braam, 1999). However, there are several reasons for believing that E2Fa-DPa-induced XETs are not required for cell expansion. First, cells divide more frequently in the E2Fa-DPa plants, but the overall cell size is smaller in transgenic than in control plants; so, no overall increase in expansion rates is needed. Second, no induction is seen of genes with a known role in cell expansion, such as expansins. Therefore, the hydrolytic activity of the XETs might rather be required to incorporate the newly synthesized cell walls formed during cytokinesis into the existing cell wall structure. Alternatively, because XET activity has been shown to be involved in the postgerminative mobilization of xyloglucan storage reserves in Nasturtium cotyledons (Farkas et al., 1992; Fanutti et al., 1993), induction of XETs in E2Fa-DPa plants might be related to polysaccharide breakdown to serve the metabolic and energy needs that are required to synthesize new nucleotides (see below).

Interestingly, two XETs can be identified in the set of twofold-or-more down-regulated genes. These XETs are more related to each other than to the induced XET genes. This differential response of XETs toward the E2Fa-DPa-induced phenotypes suggests that plant XETs can be classified into at least two different functional classes.

Genes involved in metabolism and biogenesis
A relatively large number of genes involved in metabolism and biogenesis were found in both the up-regulated and down-regulated gene groups. Most remarkable is the induction of genes involved in nitrogen assimilation, such as nitrate reductase (NIA2), glutamine synthetase (GS), and glutamate synthase (GOGAT) (Fig. 3). Although not present on the microarray, the nitrite reductase (NiR) gene was found to be induced as well in the transgenic lines, as demonstrated by RT-mediated PCR analysis (Fig. 2). Nitrogen and nitrite reductase catalyze the first two steps in the nitrogen assimilation pathway, whereas GS and GOGAT are involved both in the primary assimilation of nitrogen and the reassimilation of free ammonium. This mechanism supplies the plant with all nitrogen needed for the biosynthesis of amino acids and other nitrogen-containing compounds.

There are other indications that the nitrogen metabolism is altered in the E2Fa-DPa plants; these include the modification of genes homologous to genes expressed during the formation of nitrogen-fixing nodules in Medicago sativa (MTN3 and a nodulin-like gene), and the down-regulation of genes involved in sulfur assimilation (two different genes encoding adenylylsulfate reductase [APR] and a putative adenosine phosphosulfate kinase). Genes involved in sulfur assimilation have been shown before to be transcriptionally down-regulated during nitrogen deficiency (Koprikova et al., 2000).

The altered expression of genes involved in nitrogen assimilation and metabolism in the E2Fa-DPa transgenic plants might reflect the need for nitrogen for the nucleotide biosynthesis, because purine and pyrimidine bases are rich in nitrogen. If nitrogen assimilation were indeed stimulated by E2Fa-DPa overexpression, two requirement should be fulfilled. Firstly, there should be enough \( \alpha \)-ketoglutarate to act as an acceptor molecule for ammonium (Lancien et al., 2000)
and secondly, because assimilation of nitrogen is energy consuming, the rate of reductant production should be higher in the E2Fa-DPa transgenic than in the wild-type plants.

Our microarray data suggest that in the accumulation of α-ketoglutarate in E2Fa-DPa-overexpressing plants is stimulated in different ways. First, α-ketoglutarate production is improved by increased photosynthetic activity, as indicated by the 4.7-fold up-regulation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Fig. 3), with accumulation of glyceraldehyde-3-phosphate as a result. Glyceraldehyde-3-phosphate can be converted into fructose-1,6-bisphosphate by fructose bisphosphate aldolase. However, a sixfold down-regulation of the fructose bisphosphate aldolase gene rather suggests the conversion of glyceraldehyde-3-phosphate into pyruvate, which can be converted into α-ketoglutarate in the citrate cycle. The preferential conversion of glyceraldehyde-3-phosphate to pyruvate fits the increased need for amino acids rather than for sugars to drive nucleotide biosynthesis (Fig. 3).

A second source of α-ketoglutarate can be provided by the glyoxylate cycle. In E2Fa-DPa-overproducing plants we observed a 3.1-fold increase in expression of isocitrate lyase, suggesting an increased lipid turnover. Isocitrate lyase activity cleaves isocitrate into glyoxylate and succinate (Fig. 3). Whereas the produced glyoxylate can be converted into glycine, which is also required for de novo nucleotide biosynthesis, succinate can be converted into α-ketoglutarate in the citrate cycle. A 2.3-fold decrease in the expression of the fumarate reductase precursor even indicates an increase in chlorophyll biosynthesis (Fig. 3).

Reductant in plants mainly originates from photosynthetic electron transport in leaves. Corresponding with the increased need for reductant, several components of the chloroplast electron transport chain and associated ATP-synthesizing apparatus, such as cytochrome B6, a photosystem II subunit, and the ATPase ε subunit, are up-regulated in the E2Fa-DPa transgenic plants. Increased expression of the protochlorophyllide reductase precursor even indicates an increase in chlorophyll biosynthesis.

E2Fa-DPa plants may suffer from nitrogen starvation that has an impact on amino acid biosynthesis. Three different amino acid aminotransferases are down-regulated in the E2Fa-DPa plants. Shortage of nitrogen-rich amino acids is also evident from the reduced expression of genes encoding vegetative storage proteins (VSP1 and VSP2) and ERD10, a protein with a compositional bias toward glutamate (Kiyosue et al., 1994). Additional evidence for amino acid shortage comes from the down-regulation of a myrosinase-binding protein and the cytochrome P450 monoxygenase CYP83A1. Both proteins are involved in the biosynthesis of glucosinolates, nitrogen- and sulfur-containing products derived from amino acids (Wittstock and Halkier, 2002).

### Promoter analysis of E2Fa-DPa-regulated genes

The DNA-binding domains of the E2F and DP proteins are highly conserved between plants and mammals and, correspondingly, plant E2F-DP proteins have been shown by the technique of electrophoresis mobility shift assay to bind to the same canonical DNA-binding site as their mammalian counterparts (Albani et al., 2000; Ramirez-Parra and Gutierrez, 2000; de Jager et al., 2001). Furthermore, these E2F-binding sites regulate the expression of several plant genes involved in DNA synthesis (Kosugi and Ohashi, 2002a; Chabot et al., 2000; Castellano et al., 2001; Egelkroft et al., 2001; Stevens et al., 2002).

To distinguish between the putatively direct target genes of E2Fa-DPa and the secondarily induced genes, the first 500 bp upstream of the ATG start codon of the genes with 2-fold or higher change in expression were scanned for the presence of an E2F-like-binding site matching the (A/T)TT(G/C)(G/C)(G/C)(G/C)(G/C) sequence, which corresponds to all the different E2F-2P-binding motifs that have been described in plants. Of all the different permutations only the TTTCCCGC and TTTGCCGC elements were enriched significantly (P < 0.01) in the set of E2Fa-DPa-upregulated genes, suggesting these are the preferred binding site of the E2Fa-DPa complex (Table 3). Moreover, six out of eight target genes containing one or more of these elements belong to the group of genes involved in DNA replication and modification. The observation that not all genes that enclose this DNA sequence in their promoter are induced upon E2Fa-DPa overexpression suggests that the presence of the TTTCCCGC or TTTGCCGC motif is not the only element to

| E2F motif | All genes (4390)* | Upregulated genes (84) | Downregulated genes (104) |
|-----------|------------------|------------------------|--------------------------|
| TTTCCCCC  | 49               | 2                      | 1                        |
| TTTCCCCG  | 31               | 1                      | 1                        |
| TTTCCCCC  | 46               | 5                      | 0                        |
| TTTCCCCG  | 61               | 1                      | 1                        |
| TTTGCCC   | 16               | 0                      | 0                        |
| TTTGCC    | 76               | 2                      | 0                        |
| TTTGGCC   | 19               | 2                      | 0                        |
| TTTGCG    | 30               | 2                      | 1                        |
| TTTGCC    | 35               | 1                      | 1                        |
| TTTGCC    | 13               | 0                      | 0                        |
| TTTGGC    | 24               | 0                      | 1                        |
| TTTGCC    | 34               | 0                      | 0                        |
| TTTGCC    | 54               | 0                      | 1                        |
| TTTGGCC   | 38               | 0                      | 3                        |
| TTTGCC    | 18               | 1                      | 0                        |
| TTTGGCC   | 47               | 4                      | 0                        |
| ATTCCCCC  | 14               | 0                      | 2                        |
| ATTCCCCG  | 21               | 0                      | 0                        |
| ATTCCCG   | 11               | 1                      | 0                        |
| ATTCCCG   | 23               | 0                      | 0                        |
| ATTCCCG   | 10               | 0                      | 0                        |
| ATTCCCG   | 42               | 0                      | 0                        |
| ATTCCCG   | 13               | 1                      | 0                        |
| ATTCCCG   | 9                | 0                      | 1                        |
| ATTCCCG   | 14               | 0                      | 1                        |
| ATTCCCG   | 6                | 0                      | 0                        |
| ATTCCCG   | 15               | 0                      | 0                        |
| ATTCCCG   | 0                | 0                      | 0                        |
| ATTCCCG   | 42               | 0                      | 2                        |
| ATTCCCG   | 13               | 0                      | 1                        |
| ATTCCCG   | 12               | 2                      | 0                        |
| ATTCCCG   | 28               | 0                      | 0                        |
| Total     | 864              | 25                     | 17                       |

*Promoters of mitochondrial and chloroplastic genes were omitted from this analysis.
make a gene responsive toward E2Fa-DPa, and that E2Fa-DPa may cooperate with other factors to activate transcription. Alternatively, the promoters of non-responsive genes might be shielded with other transcription factor complexes. A putative candidate is the E2Fc protein which, in analogy with the mammalian E2F6 protein, lacks a strong transactivation domain (del Pozo et al., 2002). Alternative candidates are the recently discovered DEL proteins, proven to bind as monomers to the canonical E2F-binding site (Kosugi and Ohashi, 2002b; Mariconti et al., 2002). Because of a lack of transcriptional activation domain, the DEL proteins are postulated to act as repressors of E2F-DP-regulated genes by competing for the same binding site.

It is not excluded that genes without an E2F-like-binding site are not directly activated by E2Fa-DPAs. Chromatin immunoprecipitation experiments have shown that mammalian E2F factors can bind to promoters without a clear E2F recognition motif (Kiyosue et al., 1994), suggesting that E2F-DP might recognize non-canonical binding sites, or might be recruited by promoters through the association of other factors. In this respect, the Chlorella vulgaris nitrate reductase gene, of which the Arabidopsis homologue was shown here to be induced by E2F-DPAs, binds an E2F-DP complex, although a clear consensus binding site is lacking (Cannons and Shiflett, 2001).

E2Fs can activate as well as repress promoter activity (Trimarchi and Lees, 2002). In the PCNA, MCM3 and RNR2 promoters, E2F sequences have been identified that act as a negative regulatory element during development (Chabouté et al., 2000; Egelkrodt et al., 2001; Stevens et al., 2002). In the set of down-regulated genes, no particular enrichment of a specific E2F sequence could be seen (Table 3). Therefore, the data suggest that the E2Fa-DPa complex works as a transcriptional activator and that other E2F-DP complexes are involved in E2F-mediated transcriptional repression.

Conclusions

Microarray analysis of E2Fa-DPa-overexpressing lines identified a cross-talking genetic network between DNA replication, nitrogen assimilation and photosynthesis. The putatively direct E2Fa-DPα target genes as identified by the presence of an E2F-DP-binding site, belong to the group of genes involved in DNA synthesis, whereas the secondarily induced genes are mainly linked to nitrogen assimilation. In a recently published microarray experiment in which the periodic expression of genes during the cell cycle was monitored, genes with a role in nitrogen assimilation (aspartate aminotransferase and a nitrate transporter) were found to be specifically expressed during the S phase (Menges et al., 2002). Because purine and pyrimidine bases are nitrogen rich, we postulate that induction of nitrogen assimilation genes during DNA synthesis in wild-type and E2Fa-DPα transgenic plants is required to supply enough nitrogen for nucleotide biosynthesis. However, in the EFα-DPα transgenic plants, increased nitrogen assimilation most probably does not meet all the nucleotide biosynthesis needs, as seen by the expression modulation of many genes involved in nitrogen and carbohydrate metabolism. The drain of nitrogen from essential biosynthetic pathways to the nucleotide biosynthesis pathway is expected to affect other aspects of plant metabolism, as can be seen from the reduced expression of vegetative storage protein genes and genes involved in amino acid biosynthesis. This altered metabolism might, at least in part, contribute to the growth arrest observed in E2Fa-DPα transgenic plants.

The exact regulatory pathways and factors controlling the nitrogen assimilation pathway in plants are still unknown. In addition to the genes involved in DNA replication and metabolism, our data contain a relatively large number of genes with unspecified function (Tables 1 and 2). For instance, a GATA zinc-finger-encoded gene with a still unknown function is found between the up-regulated regulatory genes. This gene might encode the ortholog of the Neurospora crassa nit-2 protein that has been shown to positively regulate expression of the nitrate reductase gene (Fu and Marzluf, 1990). Other regulatory genes modified in the E2Fa-DPa plants encode protein kinases and several putative receptor kinases. These genes might include some novel key regulatory components in the process of nitrogen assimilation or regulation of efficient nitrogen usage. It will be of great interest to analyze their role in nitrogen assimilation, metabolism and plant growth.

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References

Albani, D., Mariconti, L., Ricagno, S., Pittlo, L., Moroni, C., Helin, K. and Cella, R. (2000). DcE2F, a functional plant E2F-like transcriptional activator from Daucus carota. J. Biol. Chem. 275, 19258-19267.

Campbell, P. and Braam, J. (1999). Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions. Trends Plant Sci. 4, 361-366.

Cannons, A. C. and Shiflett, S. D. (2001). Transcriptional regulation of the nitrate reductase gene in Chlorella vulgaris: identification of regulatory elements controlling expression. Curr. Genet. 40, 128-135.

Castellano, M. M., del Pozo, J. C., Ramirez-Parrá, E., Brown, S. and Gutierrez, C. (2001). Expression and stability of Arabidopsis CDC6 are associated with endoreplication. Plant Cell 13, 2671-2686.

Chabouté, M.-E., Clément, B., Sékine, M., Phililips, G. and Chambet-Gigot, N. (2000). Cell cycle regulation of the tobacco ribonucleotide reductase small subunit gene is mediated by E2F-like elements. Plant Cell, 1987-1999.

de Jager, S. M., Menges, M., Bauer, U.-M. and Murray, J. A. H. (2001). Arabidopsis E2F1 binds a sequence present in the promoter of S-phase-regulated gene AtCDC6 and is a member of a multigene family with differential activities. Plant Mol. Biol. 47, 555-568.

De Veylder, L., Beeckman, T., Beeemster, G. T. S., de Almeida Engler, J., Ormenese, S., Maes, S., Naudts, M., Van Der Schueren, E., Jacqmard, A., Engler, G. and Inzé, D. (2002). Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J. 21, 1360-1368.

del Pozo, J. C., Boniotti, M. B. and Gutierrez, C. (2002). Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCFASK2 pathway in response to light. Plant Cell 14, 3057-3071.

Egelkrodt, E. M., Robertson, D. and Hanley-Bowdoin, L. (2001). Proliferating cell nuclear antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves. Plant Cell 13, 1437-1452.

Fanutti, C., Gidley, M. J. and Reid, J. S. G. (1993). Action of a pure...
Microarray analysis of E2Fa-DPa plants 4259

Menges, M., Hennig, L., Gruissem, W. and Murray, J. A. H. (2002). Cell cycle-regulated gene expression in Arabidopsis. J. Biol. Chem. 277, 41987-42002.

Müller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, E., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D. and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. 15, 267-285.

Porceddu, A., De Veylder, L., Hayes, J., Van Montagu, M., Inzé, D. and Mironov, V. (1999). Mutational analysis of two Arabidopsis thaliana cyclin-dependent kinases in fission yeast. FEBS Lett. 446, 182-188.

Puskás, L. G., Zvara, A., Hackler, L. J. and Van Hummelen, P. (2002). RNA amplification results in reproducible microarray data with slight ratio bias. Biotechniques 32, 1330-1341.

Ramirez-Parra, E. and Gutierrez, C. (2000). Characterization of wheat DP, a heterodimerization partner of the plant E2F transcription factor which stimulates E2F-DNA binding. FEBS Lett. 486, 73-78.

Ramirez-Parra, E., Fründt, C. and Gutierrez, C. (2003). A genome-wide identification of E2F-regulated genes in Arabidopsis. Plant J. 33, 801-811.

Ramirez-Parra, E., Xie, Q., Boniotti, M. B. and Gutierrez, C. (1999). The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G1/S regulators. Nucleic Acids Res. 27, 3527-3533.

Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A. and Dynlacht, B. D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. Genes Dev. 16, 245-256.

Sekine, M., Ito, M., Uemukai, K., Maeda, Y., Nakagami, H. and Shimmyo, A. (1999). Isolation and characterization of the E2F-like gene in plants. FEBS Lett. 460, 117-122.

Stevens, R., Mariconti, L., Rossignol, P., Perennes, C., Cella, R. and Bergounioux, C. (2002). Two E2F sites in the Arabidopsis MCM7 promoter have different roles in cell cycle activation and meristematic expression. J. Biol. Chem. 277, 32978-32984.

Trimarchi, J. M. and Lees, J. A. (2002). Sibling rivalry in the E2F family. Nature Rev. Mol. Cell. Biol. 3, 11-20.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. (1988). Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85, 5536-5540.

Vandepoele, K., Rues, J., De Veylder, L., Roué, P., Rombouts, S. and Inzé, D. (2002). Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell 14, 903-916.

Weinmann, A., Bartley, S. M., Zhang, T., Zhang, M. Q. and Farnham, P. J. (2001). Use of chromatin immunoprecipitation to clone novel E2F target promoters. Mol. Cell. Biol. 21, 6820-6832.

Wittstock, U. and Halkier, B. A. (2002). Glucosinolate research in the Arabidopsis era. Trends Plant Sci. 7, 263-270.

Wollinger, R. D., Gibson, G., Wollinger, E. D., Bennett, L., Hamadeh, H., Buschel, P., Afschari, C. and Paules, R. S. (2001). Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. 8, 625-637.

Yang, Y. H., Dudoit, S., Luu, P., Lin, D. M., Peng, V., Ngai, J. and Speed, T. P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30, e15.

Farkas, V., Sulova, Z., Stratilova, E., Hanna, R. and Maclachlan, G. (1992). Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. Arch. Biochem. Biophys. 298, 365-370.

Fu, Y.-H. and Marzluf, G. A. (1990). nit-2, the major positive-acting nitrogen regulatory gene of Neurospora crassa, encodes a sequence-specific DNA-binding protein. Proc. Natl. Acad. Sci. USA 87, 5331-5335.

Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M. and Nevins, J. R. (2001). Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol. Cell. Biol. 21, 4684-4699.

Kel, A. E., Kel-Margoulis, O. V., Farnham, P. J., Bartley, S. M., Wingender, E. and Zhang, M. Q. (2001). Computer-assisted identification of cell cycle-related genes: new targets for E2F transcription factors. J. Mol. Biol. 309, 99-120.

Kiyosue, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994). Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in Arabidopsis thaliana L.: identification of three ERDs as HSP cognate genes. Plant Mol. Biol. 25, 791-798.

Koprivova, A., Suter, M., Op den Camp, R., Brunold, C. and Kopriva, S. (2000). Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiol. 122, 737-746.

Kosugi, S. and Ohashi, Y. (2002a). E2F sites that can interact with E2F proteins cloned from rice are required for meristematic tissue-specific expression of rice and tobacco proliferating cell nuclear antigen promoters. Plant J. 29, 45-59.

Kosugi, S. and Ohashi, Y. (2002b). E2Fs, E2F-like repressors of Arabidopsis that bind to E2F sites in a monomeric form. J. Biol. Chem. 277, 16553-16558.

Lancien, M., Gadal, P. and Hodges, M. (2000). Enzyme redundancy and the importance of 2-oxoglutamate in higher plant ammonium assimilation. Plant Physiol. 123, 817-824.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, M., Haas, H. and Loidl, P. (2002). PlantCare: a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 30, 325-327.

Lusser, A., Eberharter, A., Loidl, A., Goralič-Schramel, M., Horngacher, M., Haas, H. and Loidl, P. (1999). Analysis of the histone acetyltransferase B complex of maize embryos. Nucleic Acids Res. 27, 4427-4435.

Magyar, Z., Atanassova, A., De Veylder, L., Rombouts, S. and Inzé, D. (2000). Characterization of two distinct DP-related genes from Arabidopsis thaliana. FEBS Lett. 456, 79-87.

Mariconti, L., Pellegrini, B., Cantoni, R., Stevens, R., Bergounioux, C., Cella, R. and Albani, D. (2002). The E2F family of transcription factors from Arabidopsis thaliana. Novel and conserved components of the retinoblastoma/E2F pathway in plants. J. Biol. Chem. 277, 9911-9919.

Medford, J. I., Elmer, J. S. and Klee, H. J. (1991). Molecular cloning and characterization of genes expressed in shoot apical meristems. Plant Cell 3, 359-370.

Menges, M. and Murray, J. A. H. (2002). Synchronous Arabidopsis suspension cultures for analysis of cell-cycle gene activity. Plant J. 20, 203-212.

xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1→4)-β-D-glucanase) from the cotyledons of germinated nasturtium seeds. Plant J. 3, 691-700.