RESEARCH PAPER

Expression differences between normal and indeterminate maize suggest downstream targets of ID1, a floral transition regulator in maize

Viktoriya Coneva1, Tong Zhu2 and Joseph Colasanti1,*

1 Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1
2 Syngenta Biotechnology Inc., 3054 Cornwallis Road, Research Triangle Park, NC 27709, USA

Received 16 July 2007; Revised 21 August 2007; Accepted 22 August 2007

Abstract

The INDETERMINATE1 (ID1) transcription factor is a key regulator of the transition to flowering in maize. ID1 is expressed in immature leaves where it controls the production or transmission of leaf-derived florigenic signals. Loss-of-function id1 mutants make many more leaves than normal plants and produce aberrant flowers; however, they exhibit no obvious developmental defects in early growth stages. A maize oligonucleotide microarray was used to assess the molecular differences between immature leaves of wild-type and id1 mutant plants prior to the floral transition. This analysis revealed 55 genes with a significant 2-fold difference in expression; 22 are down-regulated and 33 are up-regulated in id1 mutants. Most prominent is a novel family of three b-glucosidase genes that are most closely related to sorghum dhurrinases. These genes, termed Zmdhr1, Zmdhr2, and Zmdhr3, are undetectable in immature leaves of id1 mutants and are expressed exclusively in normal immature leaves in a pattern identical to the ID1 gene. Other down-regulated genes include a group of four zinc finger protein-encoding genes that are unrelated to ID1. A significant number of genes up-regulated in id1 mutant immature leaves have potential roles in photosynthesis and carbon fixation, substantiating a possible connection between floral induction and assimilate partitioning. Finally, expression of these genes was compared in florally induced versus uninduced teosinte, a photoperiod-sensitive progenitor of day-neutral maize. Only a few genes showed expression differences, suggesting that ID1 acts in a novel autonomous floral induction pathway that is distinct from the photoperiod induction pathway.

Key words: Comparative expression, floral regulator, long-distance signalling, maize, molecular profiling, plant metabolism, teosinte, transcription factor.

Introduction

The transition from vegetative to reproductive development is a fundamental event in the life cycle of higher plants that is influenced by environmental and endogenous cues. Physiological studies have defined a basic floral induction circuit where florigenic signals are generated in leaves and transported via the phloem to the shoot apical meristem (SAM). The vegetative SAM receives these signals and becomes committed to reproductive development (reviewed in Corbesier and Coupland, 2006). Genetic studies in various species have defined key regulatory genes that integrate distinct floral inductive pathways. In the model plant Arabidopsis thaliana, several overlapping regulatory pathways have been established that co-ordinate environmental inductive cues, such as photoperiod and temperature, with developmental cues such as plant size and gibberellic acid hormone levels (Corbesier and Coupland, 2006). Recent studies in Arabidopsis have shown that the CONSTANS (CO) regulatory protein integrates signals from long day (LD) inductive photoperiods and the circadian clock to activate expression of the Flowering Locus T gene (FT) directly in leaves. FT protein then migrates to the SAM where it interacts with the meristem-localized transcription factor FD to activate flower identity genes and cause the transition to flowering (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007). A similar CO/FT regulatory module is proposed to be present in rice and Curcurbit

* To whom correspondence should be addressed. E-mail: jcolasan@uoguelph.ca

© 2007 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Downloaded from http://jxb.oxfordjournals.org at University of Guelph on 2 April 2009
species (Lin et al., 2007; Tamaki et al., 2007). Whether a similar system operates in other plants, and whether alternative, undiscovered inductive pathways exist has yet to be established.

In maize, the zinc finger protein encoded by the INDETERMINATE1 gene (ID1) is a key regulator of the transition from vegetative to reproductive growth. Loss-of-function id1 mutants produce many more leaves and flower much later than normal wild-type siblings (Singleton, 1946; Colasanti et al., 1998). Eventually, id1 mutants flower, but they usually lack female inflorescences or produce male flowers with vegetative characteristics. The ID1 gene product is a nuclear-localized zinc finger protein that binds a specific DNA sequence in vitro, suggesting that ID1 functions to control expression of other genes (Kozaki et al., 2004). ID1 mRNA and ID1 protein are detected only in developing leaves, and genetic studies indicate that ID1 acts specifically in leaves, suggesting that ID1 has a role in generating or transmitting a leaf-derived florigenic signal (Colasanti and Sundaesran, 2000; Wong and Colasanti, 2007). Comparative genomic analysis revealed that ID1 is the founding member of the plant-specific zinc finger IDD (ID-Domain) gene family, yet no apparent functional equivalent of ID1 exists in Arabidopsis (Colasanti et al., 2006). However, sorghum and rice, grass species that are more closely related to maize than to Arabidopsis, each has putative ID1 orthologues with leaf-specific expression patterns similar to ID1, but their roles in controlling flowering time have not been demonstrated (Colasanti et al., 2006). Further, it has not been shown whether the CO/FT regulatory module discovered in several species so far also exists in maize. Apart from ID1, the only other gene known to have a role in maize flowering is DELAYED FLOWERING1 (DLF1). Loss of DLF1 function causes plants to flower later than normal, but they do not have as severe a flowering time phenotype as id1 mutants. The DLF1 gene encodes a bZIP transcription factor that is homologous to the Arabidopsis FD protein, suggesting that DLF1 may interact with a maize FT-like partner to induce flowering (Muszynski et al., 2006). Maize also contains a large family of FT/TFL-related genes; however, none of these genes has yet been shown to have a role in flowering (Danilevskya et al., 2003; E Goerlich and J Colasanti, unpublished results).

Therefore, many questions about how ID1 controls flowering in maize remain. In particular, does ID1 activity intersect with a maize version of the CO/FT regulatory module or does a completely different system operate to control flowering in maize? Further, does a putative maize FT orthologue act as a long-distance florigenic signal, as has been suggested for other species? Genetic studies and expression analysis suggest that ID1 may act to control either the production or transmission of a leaf-derived florigenic signal. Although ID1 is detected in immature leaves exclusively, id1 mutants have no apparent defects in leaf morphology or development, nor is the vegetative growth phase of id1 plants affected. Therefore, the use of molecular profiling is an ideal way to assess important gene expression differences that exist between normal and id1 mutant plants. Here microarray profiling is used to compare molecular phenotypes of immature leaves of normal and id1 mutant maize to reveal putative direct and indirect downstream targets of ID1 activity. This is the first report of molecular profiling of the differences between flowering and non-flowering maize. Overall, 55 genes that exhibit 2-fold higher or lower levels in mutant plants were identified, including four zinc finger proteins that are activated downstream of ID1. One intriguing finding is a previously undefined set of β-glucosidase genes that are not detected or are barely detectable in id1 mutant plants. The β-glucosidase class of glycosidase hydrolase family 1 proteins are known to have diverse functions, including hormone activation, cell wall synthesis, stress tolerance, and pathogen defence (Esen, 1993). All of the β-glucosidase genes identified here are more similar to sorghum dhurrinases than to maize Zmglu1 and Zmglu2. The in vivo functions of most β-glucosidase genes are largely unknown, although the best characterized of the maize β-glucosidases, Zmglu1, was shown recently to have a possible role in cytokinin homeostasis (Kiran et al., 2006). Here three β-glucosidase genes whose expression levels and tissue specificity are associated with the ability of maize to flower are described. Possible roles of other differentially expressed genes in controlling flowering are also discussed.

Materials and methods

Plant growth and conditions

Three separate introgressions of the id1-m1 allele in two different maize inbred backgrounds were utilized for the microarray experiment. Lines 1-1@ and 2-5@ represent independent introgressions of the id1-m1 allele into the B73 inbred background, while line 62-2@ was backcrossed seven times to the W22 inbred. All seeds were sterilized in 10% bleach and planted in a 3:1 Pro- mish BX:Turface mixture. Plants were grown in the greenhouse under 14 h days at 27 °C and 10 h nights at 22 °C with fertilizer added as needed. All plants were genotyped at the three visible leaves stage (V3) by PCR as detailed by Wong and Colasanti (2007) to identify mutant and wild-type (normal) maize seedlings. Total RNA was extracted at the seven visible leaves stage (V7). For seedling root and shoot preparations, B73 inbred maize seeds were sterilized with 10% bleach and placed on wet filter paper in sterile glass Petri plates using aseptic techniques. Germination was allowed to proceed for 5 d at 25 °C in the dark. For expression experiments 2-5@ introgressed plants grown under greenhouse conditions were used. Experiments with teosinte (Zea mays ssp. parviglumis) were conducted in growth chambers under long day (LD) and short day (SD) regimes. All plants were grown in LD conditions (14 h day/10 h night) until they had made four visible leaves. Half of the plants were then transferred to SD conditions (10 h day/14 h night). All teosinte plants were harvested for RNA extraction 2 weeks after transfer to their respective light regimes.
Preparation of samples for microarray analysis

Immature leaves were excised from V7 stage plants by completely removing mature leaves 1–5 and obtaining a section of 7 cm starting 1 cm above the SAM. Three mutants (id1/id1) and three heterozygotes (id1/+id1) from lines 1-1 and 2-5, as well as two mutant and two heterozygous 62-2 plants were used. Total RNA was extracted from immature leaf tissue using TRIZOL (Sigma) as described by Colasanti et al. (1998). A Qiagen RNeasy kit (Qiagen, K7404) was used for total RNA clean-up. The resulting total RNA was quantified spectrophotometrically, precipitated, and adjusted to a concentration of 1 mg ml⁻¹. A 40 μg aliquot of each of the 16 samples was dissolved in a storage solution consisting of 70% ethanol and 0.08 M ammonium acetate to a final volume of 1 ml, and used for hybridization to the Affymetrix 82 K maize oligo microarray.

Oligonucleotide microarray hybridization and data analysis

A Syngenta custom-designed maize GeneChip microarray, manufactured by Affymetrix, was used for this study. The GeneChip array consists of ~87 000 probe sets representing 82 000 unique maize expressed sequence tag (EST) clusters and genes, and various negative, spike, and transgenic control genes. Each probe set contains an average of 13 perfect match 25mer oligonucleotide probes. A 5 μg aliquot of total RNA from each sample was used as template for cDNA and cRNA synthesis. The cRNAs were further fragmented and labelled as previously described (Zhu et al., 2006). Labelled cRNAs were applied to the maize GeneChip microarray, and subsequent hybridization, washing, and staining were conducted according to the Affymetrix recommended protocol. The image files (*.DAT) and raw files (*.CEL) were acquired using Microarray Analysis Suite 5.0 (Affymetrix). The expression level for each gene was summarized from measurements of individual probes of a set using a custom algorithm. Briefly, individual probe values were calculated by removing the local background (average of the lowest 2% of probe values in one of the 16 sectors on the array). The expression value measured by a probe set was represented by the median of the intensity values of all probes except those with negative or zero values. The absolute calls were assigned based on the difference between the expression value of a probe set and the local noise (standard deviation of all pixels) in the probe sets and global background levels (the average of the lowest 5% of the probe set value): probe sets with a signal value above the global background plus twice the local noise were called ‘present’; probe sets with a value less than global background were called ‘absent’; and probe sets with values greater than global background but less than global background plus twice the local noise were called ‘marginal’. The resulting data were imported into GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA, USA). Microarray data were grouped into mutants and heterozygotes (wild-type), and each group was treated as a continuous parameter. The raw data were normalized to the 50th percentile per chip and to the median per gene. The data were then filtered on the expression level with a set minimum of 25, as well as on flag, i.e. only signals identified as ‘medium’ or ‘present’ were included in the analysis.

Further, a volcano plot was generated to obtain all probes with at least a 2.0-fold change value between groups and a P-value <0.05. The generated volcano plot was additionally tested by a one-way analysis of variance (ANOVA) t-test. Fold change values for a subset of genes were checked with northern blots and semi-quantitative RT-PCR as described below.

Expression analysis of β-glucosidases and select genes

For northern blots, 20 μg of total RNA was run on a MOPS-formaldehyde gel and blotted onto a Hybond-N+ membrane for nucleic acid transfer (Amersham Pharmacia Biotech). Probes were generated by amplification of SuperScript II (Invitrogen) reverse-transcribed RNA. All primers were designed to amplify identified sequences on the array and are listed in Supplementary Table S1 available at JXB online. Amplification products were gel extracted using a QIAquick gel extraction kit (Qiagen). The identity of the purified northern blot probes was verified by sequencing or restriction digestion. The probes were radioactively labelled using [α-32P]dCTP and Amersham Biosciences Ready-to-go DNA Labeling Beads (Amersham Biosciences, 27-9240-01). For expression analysis, plants were harvested at the V7 stage, leaves 1–5 were removed, and immature leaf was divided into consecutive 2 cm sections starting at the shoot apex: ‘A’, ‘B’, ‘C’, and ‘D’ (Fig. 3A). The tip of mature leaf 5 (L5) was also included in the analysis. The RNA was cleaned up using a Qiagen RNeasy kit (Qiagen, K7404), which includes a DNA removal step. cDNA was generated using oligo(dT)₁₂−₁₈ priming and SuperScript II Reverse Transcriptase (Invitrogen). Finally, Escherichia coli RNase H (Invitrogen) was used to remove residual RNA after reverse transcription. After quantifying and adjusting the cDNA concentration, 80, 8, 0.8, and 0.08 ng of template were used in a dilution series with each set of primers in order to adjust the RT-PCRs within a linear range. All RT-PCR experiments were repeated at least three times to confirm reproducibility. All primers were tested on cDNA and genomic DNA templates to confirm that they spanned intron sequences (Supplementary Fig. S2 available at JXB online).

Alignment and phylogeny of predicted maize β-glucosidase proteins

Protein sequences for ZmDh1r1, ZmDh1r2, and ZmDh1r3 were predicted based on full EST sequences and aligned with protein sequences for Zmglu1 (CAA52293), Zmglu2 (AAD09850), and Sorghum bicolor glycosyl hydrolase dhurrinase (AAC49177) in BioEdit 7.0 using a BLOSUM62 similarity matrix (Hall, 1999). The alignment was transferred to MEGA version 3.1 (Kumar et al., 2004) and a neighbour-joining (NJ) tree was constructed with 500 bootstraps, pair-wise deletion of gaps, and a Poisson correction model. Arabidopsis thaliana glycosyl hydrolase family 1 protein (At5g44640) was used as an outgroup.

Results

Identification of expression differences between normal and id1 mutant maize plants

Although id1 mutants are severely impaired in their ability to flower, up until the point of floral transition they are virtually identical in their growth rate and morphology to normal maize plants when grown under standard growth chamber conditions (Fig. 1A). Further, ID1 activity appears to be spatially confined to a particular tissue, i.e. ID1 mRNA and ID1 protein are present in the distal regions of immature, developing leaves and absent from the SAM and nascent leaf primordia, as well as from photosynthetically active mature green leaves (Colasanti et al., 1998; Wong and Colasanti, 2007). Temporally, ID1 transcript and protein are confined to immature leaves at all stages of development, but the overall level of ID1 mRNA increases as the plant approaches the transition to flowering. Therefore, differential expression profiling was
thought to be well suited for revealing transcriptional differences between flowering and non-flowering maize. For this study, expression differences between immature leaves of mutant and wild-type plants were examined just prior to the transition to flowering. Also, by restricting the analysis to immature leaves at the transition stage, it was hoped to increase the chances of finding genes that are direct targets of the ID1 transcription factor.

Plants segregating the id1-m1 null mutation, which is caused by a Ds2 transposable element insertion into an exon of the ID1 gene (Colasanti et al., 1998), were used for all experiments. The id1-m1 allele was backcrossed into inbreds B73 and W22 to create three lines for use in the microarray analysis; lines 1-1 and 2-5 were derived from independent introgressions of the id1-m1 allele into the B73 background, while 62-2 contains the id1-m1 allele introgressed into a W22 inbred background (see Materials and methods). The use of introgression lines and different inbred backgrounds further reduces the likelihood of expression differences unrelated to flowering. Total RNA was prepared from the central cylinder of immature leaves of id1 mutant and ID1+ plants (hereafter referred to as ‘normal’ or ‘wild-type’) that had made 7–8 visible leaves, i.e. the V7/V8 stage (Fig. 1A). For B73 and W22 seedlings, the V7/V8 stage corresponds to the point in plant development when vegetative growth is completed and no further leaves are initiated (Colasanti et al., 1998; Wong and Colasanti, 2007). Triplicate samples were taken from the 1-1 and 2-5 lines and duplicates from 62-2 plants, for a total of eight mutants and eight wild-type heterozygous plants. Maize id1/ID1+ heterozygotes show no differences in flowering compared with ID1+/ID1+ homozygous plants (J. Colosanti, unpublished data). At this early stage of growth there are no visible morphological differences between mutant and normal plants, thus minimizing gene expression differences not attributable to flowering.

The custom-designed Affymetrix oligonucleotide array interrogated here contains 82 000 probe sets representing ~87 000 genes and unique EST clusters. These probe sets cover a significant portion of the maize transcriptome, with 50 058 displaying high similarities to known proteins. To prevent overassembly of sequences and subsequent misassembly of probe sets, a conservative approach was used to assemble the sequences during array design. As a consequence, redundant probe sets representing the same gene are observed and combined during microarray data analysis. To identify the gene sets with the greatest reproducible expression differences, a 2.0-fold change threshold and P-values <0.05 were set as criteria.
Table 1. Summary of 55 chip probes with a minimum of 2.0-fold expression difference between mutant and wild-type plants

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Fold change</th>
<th>Annotation (putative)</th>
<th>Accession</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zm019329</td>
<td>15.90</td>
<td>Zmdhr1 β-glucosidase 1 (Zm)</td>
<td>MRT4577.20572C</td>
<td>3.25E-10</td>
</tr>
<tr>
<td>Zm022521</td>
<td>12.20</td>
<td>Zmdhr2 β-glucosidase 2 (Zm)</td>
<td>P45285.1p</td>
<td>4.64E-11</td>
</tr>
<tr>
<td>Zm059623</td>
<td>6.01</td>
<td>CHY zinc finger family protein (Os)</td>
<td>AAP54090*</td>
<td>0.00769</td>
</tr>
<tr>
<td>Zm022542</td>
<td>4.88</td>
<td>Zmdhr3 β-glucosidase 3 (Zm)</td>
<td>MRT4577.20571C</td>
<td>7.55E-09</td>
</tr>
<tr>
<td>Zm018613</td>
<td>4.70</td>
<td>CSL zinc finger domain-containing protein (At)</td>
<td>AT2G15910*</td>
<td>0.0131</td>
</tr>
<tr>
<td>Zm061836</td>
<td>4.61</td>
<td>PIFI post-illumination chlorophyll fluorescence increase protein (Zm)</td>
<td>ABI51593</td>
<td>4.89E-07</td>
</tr>
<tr>
<td>ZMU44087</td>
<td>4.06</td>
<td>Zmglu2 β-glucosidase precursor (Zm)</td>
<td>AAD09850</td>
<td>1.21E-05</td>
</tr>
<tr>
<td>Zm014771</td>
<td>3.52</td>
<td>No description (Os)</td>
<td>Os1g137580*</td>
<td>0.0138</td>
</tr>
<tr>
<td>Zm035896</td>
<td>3.51</td>
<td>Retrotransposon protein (Os)</td>
<td>Os1g0768200a</td>
<td>3.36E-06</td>
</tr>
<tr>
<td>Zm033525</td>
<td>3.13</td>
<td>CHY zinc finger domain protein1 (Os)</td>
<td>AAV64189</td>
<td>0.00655</td>
</tr>
<tr>
<td>Zm044841</td>
<td>2.72</td>
<td>Kinesin heavy chain (Zm)</td>
<td>AAK91821</td>
<td>0.00201</td>
</tr>
<tr>
<td>Zm047669</td>
<td>2.72</td>
<td>Small heat shock like protein (Os)</td>
<td>Os1g0437700a</td>
<td>3.36E-06</td>
</tr>
<tr>
<td>AF058757</td>
<td>2.63</td>
<td>Zinc finger protein ID1 (Zm)</td>
<td>AAC18941</td>
<td>3.45E-07</td>
</tr>
<tr>
<td>Zm019317</td>
<td>2.58</td>
<td>Acyl-desaturase, plastid precursor (Os)</td>
<td>Os0g0199400a</td>
<td>0.0204</td>
</tr>
<tr>
<td>Zm021672</td>
<td>2.44</td>
<td>Peptide transporter PTR2 (Zm)</td>
<td>Os1g0469900a</td>
<td>0.00201</td>
</tr>
<tr>
<td>Zm022540</td>
<td>2.29</td>
<td>Ring finger and CHY zinc finger domain-containing protein1 (Os)</td>
<td>Os1g0456800a</td>
<td>0.00196</td>
</tr>
<tr>
<td>Zm022003</td>
<td>2.27</td>
<td>Auxin-induced protein PCNT115 (Os)</td>
<td>ABG66079*</td>
<td>0.0116</td>
</tr>
<tr>
<td>Zm031273</td>
<td>2.25</td>
<td>Zinc finger, H3HC4 type protein family (Os)</td>
<td>Os1g0481400a</td>
<td>0.0259</td>
</tr>
<tr>
<td>Zm031351</td>
<td>2.23</td>
<td>Ribosomal protein S19 (Os)</td>
<td>BAC19887*</td>
<td>0.000933</td>
</tr>
<tr>
<td>Zm006228</td>
<td>2.20</td>
<td>Pathogenesis-related thaumatin family protein (Zm)</td>
<td>AAV64186</td>
<td>0.0459</td>
</tr>
<tr>
<td>Zm090933</td>
<td>2.19</td>
<td>Putative RNA-directed RNA polymerase I (Os)</td>
<td>BAC07203*</td>
<td>0.0259</td>
</tr>
<tr>
<td>Zm071638</td>
<td>2.12</td>
<td>Phosphate-induced protein phi-1 (Os)</td>
<td>BAD17079*</td>
<td>0.00031</td>
</tr>
</tbody>
</table>

**Down-regulated genes**

**Up-regulated genes**

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Fold change</th>
<th>Annotation (putative)</th>
<th>Accession</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zm005168</td>
<td>0.35</td>
<td>Xyloglucan endotransglycosylase/hydrolase protein 15 precursor (Os)</td>
<td>Os04g0604300*</td>
<td>0.0185</td>
</tr>
<tr>
<td>Zm064890</td>
<td>0.35</td>
<td>Expressed protein (Os)</td>
<td>Os1g0957200a</td>
<td>0.00607</td>
</tr>
<tr>
<td>Zm030367</td>
<td>0.39</td>
<td>D-glucosidase precursor (XL)</td>
<td>Os04g0590100a</td>
<td>0.0231</td>
</tr>
<tr>
<td>Zm021422</td>
<td>0.39</td>
<td>D-glucosidase 3 (XL)</td>
<td>Os04g0590100a</td>
<td>0.0231</td>
</tr>
<tr>
<td>MZENDMEX</td>
<td>0.40</td>
<td>NADP-dependent malic enzyme (Zm), plastid precursor</td>
<td>P16243</td>
<td>0.0204</td>
</tr>
<tr>
<td>Zm017950</td>
<td>0.40</td>
<td>Expressed protein (Os)</td>
<td>Os3g0744700a</td>
<td>0.0459</td>
</tr>
<tr>
<td>Zm002995</td>
<td>0.40</td>
<td>Fructose-bisphosphate aldolase, plastid precursor (ALDP) (Os)</td>
<td>Q40677*</td>
<td>0.000889</td>
</tr>
<tr>
<td>Zm013248</td>
<td>0.40</td>
<td>Senescence-associated protein DH (Zm)</td>
<td>AAV31120</td>
<td>0.0313</td>
</tr>
<tr>
<td>Zm034850</td>
<td>0.41</td>
<td>α-Amylase inhibitors, seed storage protein family protein (Os)</td>
<td>AAV31120</td>
<td>0.0313</td>
</tr>
<tr>
<td>Zm03681</td>
<td>0.43</td>
<td>α-Amylase inhibitors, seed storage protein family protein (Os)</td>
<td>AAS07276*</td>
<td>0.0259</td>
</tr>
<tr>
<td>Zm006657</td>
<td>0.44</td>
<td>Photosystem I protein-like protein (Os)</td>
<td>BAC833352.1*</td>
<td>0.0279</td>
</tr>
<tr>
<td>Zm031706</td>
<td>0.44</td>
<td>Expressed protein (Os)</td>
<td>Os2g0740600a</td>
<td>0.0211</td>
</tr>
<tr>
<td>Zm032848</td>
<td>0.44</td>
<td>Mucin-2 precursor (Os)</td>
<td>Os08g0478000a</td>
<td>0.0462</td>
</tr>
<tr>
<td>MZEPOD</td>
<td>0.45</td>
<td>Chain A, pyruvate phosphate dikinase (Zm)</td>
<td>1VBGA</td>
<td>0.033</td>
</tr>
<tr>
<td>Zm014577</td>
<td>0.45</td>
<td>Type II light-harvesting chlorophyll a/b-binding protein (Os)</td>
<td>BAA00357*</td>
<td>0.0433</td>
</tr>
<tr>
<td>AFO84478</td>
<td>0.45</td>
<td>Ribulose bisphosphate carboxylase/oxygenase activase (Zm)</td>
<td>Q9ZT00</td>
<td>0.018</td>
</tr>
<tr>
<td>Zm00433</td>
<td>0.46</td>
<td>Serine/threonine protein kinase (Os)</td>
<td>AAS07272*</td>
<td>0.0259</td>
</tr>
<tr>
<td>Zm05951</td>
<td>0.46</td>
<td>ATP-binding protein (Os)</td>
<td>Os10g0457700a</td>
<td>0.00769</td>
</tr>
<tr>
<td>Zm021382</td>
<td>0.47</td>
<td>ATPase, coupled to transmembrane movement of substances (Os)</td>
<td>Os03g0281900a</td>
<td>0.0202</td>
</tr>
<tr>
<td>Zm032112</td>
<td>0.47</td>
<td>Expressed protein (Os)</td>
<td>Os12g0453500a</td>
<td>0.0196</td>
</tr>
<tr>
<td>Zm069047</td>
<td>0.47</td>
<td>Chalcone synthase C2 (naringenin-chalcone synthase C2) (Zm)</td>
<td>P24825</td>
<td>0.0375</td>
</tr>
<tr>
<td>ZMU43034</td>
<td>0.48</td>
<td>Nucleoside-ascorbate transporter leaf permease protein 1 (Zm)</td>
<td>Q41760</td>
<td>0.0202</td>
</tr>
<tr>
<td>Zm065912</td>
<td>0.48</td>
<td>Calmodulin-like protein (Penisutum ciliare)</td>
<td>AAK15501*</td>
<td>0.033</td>
</tr>
</tbody>
</table>
to analyse the hybridization data, and 67 hits were identified as statistically significant (Table 1, Supplementary Fig. S1 at JXB online). A false discovery rate of 0.05 was used as a cut-off criterion for all expression analysis. The total number was collapsed to 55 genes because several of the genes were represented multiple times on the array. Of these, 22 genes were down-regulated by at least 2-fold in id1 mutants compared with the wild type, while 33 were up-regulated by \( \geq 2 \)-fold. As expected, ID1 was among the 22 genes with reduced expression levels in id1 plants, with an overall fold change of 2.63 (Table 1). The level of fluorescence for the ID1 probe set on the microarray was relatively low, as expected for the expression of transcription factor genes. Furthermore, hybridization to two actin probe sets on the chip did not vary significantly between mutant and normal plants, validating the usefulness of this probe as an expression control for RT-PCR.

To characterize further genes with the greatest differences, microarray probe region sequences were used to query available EST and cDNA databases, as well as the Genomic Survey Sequence (GSS) database comprised of DNA sequences enriched for coding regions (http://www.ncbi.nlm.nih.gov/dbGSS/). BLAST searches with full-length ESTs were also used in some cases to annotate putatively 55 genes with significant expression differences (Table 1). One of the most outstanding differences is the down-regulation in id1 mutants of three unique \( \beta \)-glucosidase-related genes, with fold changes ranging from 4.9 to as much as 15.9 (Fig. 1B). These are described in more detail later. It is also intriguing to note that at least 19 of the 55 unique genes (34%) are involved in various aspects of photosynthesis and carbohydrate metabolism, and 15 of these are up-regulated in id1 mutants (Table 1). In addition to ID1, four genes encoding different classes of zinc finger-domain-containing proteins were down-regulated in id1 mutants. Two genes encode CHY-type zinc fingers: one is a CSL-type zinc finger and the other has a H3HC4 zinc-binding motif; none of these genes is similar to \( IDD \)-type genes defined by ID1 (Colasanti et al., 2006).

### Confirmation of expression differences by RT-PCR and northern analysis

The validity of expression differences detected by microarray was examined by gene-specific probes on northern blots and/or by semi-quantitative RT-PCR with intron-spanning primers (Supplementary Table S1 at JXB online) for selected genes with the greatest expression differences (Table 1). For all RT-PCR experiments, several dilutions of template cDNA were tested in order to ensure that gene amplification remained in the linear range (see Materials and methods). The prominent down-regulation of several \( \beta \)-glucosidase genes was confirmed by northern blots as well as by RT-PCR (Figs 1B and 3B). RT-PCR was also used to examine the expression of six genes identified as up-regulated in id1 mutants as well as three other genes found to be at significantly lower levels in mutant plants, in addition to four \( \beta \)-glucosidase genes (Figs 2 and 3B). In most cases RT-PCR analysis confirmed the differences detected by microarray (Figs 2B and 3B). For example, three \( \beta \)-glucosidase genes with the greatest difference of expression (\( Zmdh1r1 \), \( 2 \), and \( 3 \)) were abundant in wild-type plants, but were not detectable by RT-PCR in id1 mutants (Fig. 3B), and \( Zmdh1r1 \) and \( Zmdh2r2 \) mRNAs were not detected by northern analysis (Fig. 1B). Similarly, expression of two of the zinc finger genes, CHY and CSL, was significantly lower in id1 mutant leaves by RT-PCR and corresponded approximately to levels shown by microarray (Fig. 2A, B). In several cases, however, the magnitude of the changes observed by microarray analysis was not supported by RT-PCR. Although RT-PCR analysis of one of the \( \beta \)-glucosidase genes, \( Zmglu2 \), showed its

### Table 1. (Continued)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Fold change</th>
<th>Annotation (putative)</th>
<th>Accession</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zm004731</td>
<td>0.48</td>
<td>Phosphoribulokinase, chloroplast percursor (Os)</td>
<td>Os02g069000*</td>
<td>0.0243</td>
</tr>
<tr>
<td>Zm021787</td>
<td>0.49</td>
<td>Glycosyltransferase family 9 protein (Os)</td>
<td>NP_001061432*</td>
<td>0.00219</td>
</tr>
<tr>
<td>Zm027522</td>
<td>0.49</td>
<td>Chlorophyll a/b-binding protein type III precursor (Sl)</td>
<td>Sl04125*</td>
<td>0.0204</td>
</tr>
<tr>
<td>Zm013762</td>
<td>0.49</td>
<td>Plant integral membrane protein (Os)</td>
<td>Os05g0245300*</td>
<td>0.0243</td>
</tr>
<tr>
<td>Zm0236601</td>
<td>0.49</td>
<td>Photosystem I P700 apoprotein A2 (Saccharum hybrid)</td>
<td>YP_023479*</td>
<td>0.0202</td>
</tr>
<tr>
<td>Zm012102</td>
<td>0.50</td>
<td>Chlorophyll a/b-binding protein 6A, chloroplast precursor (Os)</td>
<td>Os06g0320500*</td>
<td>0.0355</td>
</tr>
<tr>
<td>Zm031682</td>
<td>0.50</td>
<td>Ribulose bisphosphate carboxylase small chain, chloroplast precursor (Zm)</td>
<td>P05348</td>
<td>0.0034</td>
</tr>
<tr>
<td>Zm073098</td>
<td>0.50</td>
<td>Plastocyanin, chloroplast precursor (Os)</td>
<td>Os06g0101600*</td>
<td>0.0196</td>
</tr>
<tr>
<td>Zm054116</td>
<td>0.50</td>
<td>CBL-interacting protein kinase 4 (CIPK4) (Ar)</td>
<td>AT4G14580*</td>
<td>0.0313</td>
</tr>
</tbody>
</table>

\* Full-length EST available from MaizeSeq (www.maizseq.org).
transcript levels to be significantly lower in id1 mutants (Fig. 3B), this difference did not match the 4-fold up-regulation in wild-type immature leaves as shown by microarray. Similarly the IAA β-glucosyltransferase gene (Zm003067) that was found to be up-regulated on average 2.5-fold in id1 immature leaves by microarray was shown to be only marginally higher by RT-PCR (Fig. 2B). Conversely, the PS1 protein gene (Zm000657) showed no apparent difference in any tissue by RT-PCR even though microarray analysis suggested a 2.5-fold up-regulation in id1 immature leaves. The discrepancy in these cases is probably due to cross-hybridization to other closely related members of the respective gene families. Overall, these data generally support the accuracy of the microarray profiles, but also reaffirm the importance of checking microarray results by other expression methods.
Determination of expression patterns of identified genes in other maize tissues

All microarray analyses were performed with RNA derived from immature leaves, i.e. the tissue where ID1 mRNA and its gene product are localized (Colasanti et al., 1998; Wong and Colasanti, 2007). RT-PCR was used to examine transcript levels of the 13 genes described above in other vegetative tissues of wild-type plants to determine if they shared an expression pattern similar to that of ID1, i.e. limited to immature leaves but undetectable in mature leaf blades or meristematic regions (Fig. 3B). The central stem cylinders of V7 floral transition stage wild-type B73 inbred plants, not including the leaf sheath, were sectioned into four regions as previously described (Colasanti et al., 1998). Section ‘A’ contains the entire SAM as well as the first few leaf primordia and encompasses a region from the base of the apex to 2 cm above it. Sections ‘B’, ‘C’, and ‘D’ are consecutive 2 cm long sections above section ‘A’ (Fig. 3A); these sections were found to have the highest levels of ID1 mRNA (Colasanti et al., 1998). Transcript levels were also examined in the fifth mature green leaf of V7 plants and the emerging root and shoot of 3 DAG (days after germination) seedlings (Fig. 3A).

In addition to the β-glucosidase genes (described below), the expression of several genes that are down-regulated in immature leaves of id1 mutants was analysed. Transcripts for two putative zinc finger-encoding genes as well as the post-illumination fluorescence increase (PIFI) gene were also detected at lower levels in mature green leaves of id1 mutants relative to the wild type (Fig. 2B). However, unlike ID1, these genes are present in all sections of the stem as well as in germinating shoots and root tissues. The CHY zinc finger gene mRNA is present in all tissues equally, whereas CSL zinc finger transcripts are most abundant in immature leaves and germinating roots, and less so in green leaves (Fig. 2B).

The expression of six genes that were up-regulated in id1 immature leaves according to the microarray data analysis was also profiled. As described above, higher transcript levels in id1 immature leaves for four of the six genes were clearly validated by RT-PCR. All of these genes have a putative role in photosynthesis, including malate dehydrogenase (MDH), chloroplastic NADP-dependent malic enzyme (NADP-ME), a chloroplast-specific fructose bisphosphate aldolase (FBP) precursor gene, and a Rubisco activase (RCA) gene. Interestingly, none of these genes shows a difference in expression between mutant and wild type in mature green leaves, i.e. all are present at high levels in both id1 and normal green leaves (Fig. 2B). Therefore, immature leaves of id1 mutants seem precociously to express several genes that are normally present in photosynthetically active tissues. The most striking difference was found for the MDH gene, which is barely detectable in wild-type immature leaves, but is expressed at higher levels in id1 immature leaves than in green leaves of either mutant or wild type. Similarly, the chloroplastic malic enzyme transcript is abundant in all green leaves, as well as id1 immature leaves. Overall, these results suggest an apparent shift in temporal development of the photosynthetic apparatus in id1 mutants. However, there are no obvious differences in id1 mutant plants with respect to morphology or chlorophyll levels (Wong and Colasanti, 2007; data not shown).

Not unexpectedly, none of these photosynthesis-related genes showed expression patterns similar to ID1 since all of them are expressed primarily in mature green leaves. Expression patterns of the putative FBP aldolase precursor gene (Zm002995) and the RCA precursor gene (AF084478) were very similar, with both showing high levels of expression in green leaves, as well as in the more distal C and D immature leaf sections (Fig. 2B). Transcripts of the putative maize IAA β-glucosyltransferase gene (Zm003067), which showed only slightly higher levels in id1 immature leaves, were also detected at equal levels in green leaves of both mutant and wild type (Fig. 2B).

Expression analysis of maize β-glucosidase-related genes

Microarray profiling detected four β-glucosidase-related genes that are down-regulated in immature leaves of id1 mutants (Table 1). Three β-glucosidase genes with the greatest expression differences were found to be most similar to the dhurrinase class of glucose hydrodases and are, therefore, referred to here as Zmdhr1, Zmdhr2, and Zmdhr3 (see below). Expression of the probe set for Zmdhr1 is 15.9-fold higher, on average, in normal plants relative to id1 mutants, the greatest statistically significant difference for any probe on the array, while three other β-glucosidase genes, represented by probe sets for Zmdhr2, Zmdhr3, and ZMU44087, were 12.2-, 4.9-, and 4.1-fold higher, respectively, in wild-type immature leaves relative to id1 mutants (Table 1). Northern blots, and up to 32 cycles of RT-PCR, were unable to detect transcript in id1 plants for any of these genes except ZMU44087, while high levels of expression were detected in wild-type plants (Figs 1B and 3B). Therefore, northern and RT-PCR analyses show a more dramatic difference between mutant and wild type that is not apparent from the microarray data. This is a trend that has been widely reported in microarray experiments (Clarke and Zhu, 2006).

It is interesting to note that, unlike any of the nine genes analysed and described above, the three Zmdhr β-glucosidase genes all have expression patterns that are strikingly similar to that of the ID1 gene, i.e. they are expressed in distal regions of immature leaves but undetectable in mature leaf blades or the apical regions of wild-type plants (Colasanti et al., 1998; Fig. 3B). Also...
similar to ID1, the three Zmdhr genes are undetectable or expressed at very low levels in roots and shoots of 3 DAG seedlings (Fig. 3B). The fourth β-glucosidase probe set, ZMU44087, which corresponds to the maize Zmglu2 β-glucosidase gene, is expressed in mature leaf blades of both wild-type and mutant plants, with lower but detectable expression levels in id1 mutants. Moreover, Zmglu2 transcripts are present in all sections of immature leaf as well as root and shoot of young seedlings, consistent with previous reports (Cicek and Esen, 1999). Therefore, the expression pattern of Zmglu2 is unlike that of the three Zmdhr β-glucosidase genes. The Zmglu1 gene, which is closely related to Zmglu2, was also tested and it was found that its expression does not vary between id1 mutant and wild-type plants (Fig. 3B). Therefore, the three Zmdhr genes define a new class of maize β-glucosidase genes with expression patterns that overlap with that of the ID1 gene.

Phylogenetic comparison of β-glucosidase-related genes

BLAST searches of EST, cDNA, and GSS databases with chip probe sequences allowed reconstruction of the complete coding regions for newly found differentially expressed β-glucosidase genes. An alignment of predicted β-glucosidase amino acid sequences including the three maize dhurrinase-related β-glucosidases, along with Zmglu1, Zmglu2, and sorghum Sbdhr1, revealed a high degree of similarity among the deduced protein sequences (Fig. 4A). The TFNEP and ITENG motifs, which form the catalytic site of all family 1 β-glucosidases (Czjzek et al., 2000), were found to be conserved in all β-glucosidase genes examined here (red boxes, Fig. 4A). The level of relatedness among maize β-glucosidases was examined by constructing an NJ tree based on the generated amino acid alignment (Fig. 4B). The sequences of the S. bicolor β-glucosidase dhurrinase (AAC49177) and an A. thaliana glycosyl hydrolase family 1 protein (At5g44640) were also included. Maize dhurrinase-related β-glucosidases group together as close relatives and are similarly distanced from maize Zmglu2 and Sbdhr1. Zmdhr1 and Zmdhr2 are 97% identical at the amino acid level, while Zmdhr3 shares only 75% identity with Zmdhr1, and 74% with Zmdhr2. When compared as pairs, Zmdhr1 shares 73% identity with Zmglu1 and 71% identity with Zmglu2 and Sbdhr1. Zmdhr3 shares 71% of its sequence with Zmglu1 and Zmglu2, while it is 69% identical to Sbdhr1. Zmglu1 is 89% identical to Zmglu2, while Zmglu1 and Zmglu2 proteins are about 71% identical to Sbdhr1. Therefore, Zmdhr1, Zmdhr2, and Zmdhr3 comprise a previously unidentified class of family 1 maize glycolytic hydrolases that are rather more similar to sorghum dhurrinases than to the Zmglu1 and Zmglu2 β-glucosidases.

Comparison of differentially expressed genes in florally induced and vegetatively growing teosinte

Maize (Z. mays ssp. mays) grown in temperate climates is largely day-neutral with respect to flowering time, i.e. regardless of photoperiod, the SAM will initiate a defined number of leaves and then form an inflorescence (Galinat and Naylor, 1951). In contrast, the progenitor of maize, teosinte (Z. mays ssp. parviglumis), is an obligate SD plant that requires long nights to induce flowering. The floral inducibility of teosinte was used to ascertain whether any of the genes identified in the microarray analysis show similar differences in expression in florally induced versus uninduced plants. It has been determined that 100% of teosinte plants grown under non-inductive LDs of 14 h days and 10 h nights undergo reproductive development and form flowers after being shifted to an SD photoperiod (10 h days/14 h nights) for 2 weeks (V Coneva and J Colasanti, unpublished observations). In this study, teosinte was germinated and grown under LD conditions until 4–5 leaves had formed, and then some of the plants were shifted to SD conditions to induce flowering, while others remained under LD conditions to develop vegetatively. After 2 weeks, RNA was extracted from the immature leaf cylinder of induced and uninduced teosinte plants at the V7/V8 stage and RT-PCR was performed to check relative gene expression levels. It was reasoned that changes in gene expression associated with the transition to flowering in temperate maize might also be observed in induced and uninduced teosinte. That is, the genes found to be down-regulated in id1 mutant leaves, and, therefore, whose expression is associated with the ability to flower, might be up-regulated in florally induced (SD) teosinte leaves. Conversely, genes that are up-regulated in id1 mutants, and, therefore, higher in non-flowering plants, would be expressed at lower levels in florally induced teosinte. Many of the 13 genes examined here showed no expression differences in induced and uninduced teosinte immature leaves. For example, genes expressed differently in mutant versus wild-type immature leaves, such as FBP aldolase, chloroplast NADP-ME, MDH, and RCA, showed no obvious differences in teosinte immature leaves (Figs 2C and 3C). Some genes showed the opposite patterns to what would be expected. For example, IAA β-glucosyltransferase mRNA, which was found to be marginally higher in id1 mutants, was higher in SD-grown florally induced teosinte (Fig. 2B, C), as were transcripts of the Zmdhr3 gene (Fig. 3C). Interestingly, transcripts for CHY and CSL zinc finger genes and PIFI, which are down-regulated in id1 mutants (Fig. 2B), were detected at higher levels in SD-grown, florally induced teosinte (Fig. 2C). Similarly, Zmdhr1 and Zmdhr2 transcripts appear only marginally higher in immature leaves of florally induced SD teosinte when compared with LD plants. Possible explanations of the
**Fig. 4.** (A) Alignment of known and deduced amino acid sequences of maize β-glucosidase-related genes and the *Sorghum bicolor* glycosyl hydrolase dhurrinase. Full protein sequences for Zmdhr1, Zmdhr2, and Zmdhr3 were predicted based on full EST sequences (see Table 1 for details) and aligned with protein sequences for Zmglu1 (CAA52293), Zmglu2 (AAD09850), and *S. bicolor* glycosyl hydrolase dhurrinase (AAC49177) in BioEdit 7.0 using a BLOSUM62 similarity matrix. Identity shading is shown in dark grey, while highly similar positions are depicted in light grey. The TFNEP and ITENG motifs, which form the catalytic site of all family 1 β-glucosidases, are boxed in black. Arrows point to key amino acid residues required for dhurrin cleavage: S-481, S-482, and R-487. (B) A neighbour-joining (NJ) tree comparing β-glucosidase-related genes from *Z. mays* (Zm), *S. bicolor* (Sb), and *Z. diploperennis* (Zm).
significance of these findings from this preliminary analysis with teosinte are discussed below.

**Discussion**

The maize ID1 gene encodes a transcriptional regulator that acts in leaves to mediate the transition to flowering in maize. Molecular profiling in immature leaves was used to examine the expression differences between normal maize and id1 mutant plants in order to identify potential downstream targets of ID1. There are two main reasons for taking this approach. First, prior to the transition to flowering, normal maize plants are indistinguishable from id1 mutants. Therefore, any observed expression differences are more probably associated with the inability of id1 mutant leaves to produce or transmit the florigenic signal rather than the formation of reproductive structures. Secondly, by focusing on immature developing leaves, the tissue where ID1 is expressed, it was hoped to reveal downstream targets that are more directly affected by ID1 function.

Relatively few expression differences are detected between immature leaves of normal and id1 mutant maize plants

A total of 55 unique genes with a minimum of 2-fold differential expression were identified in immature leaves of normal and id1 plants. It is conceivable that genes with expression differences of <2-fold may have roles in flowering, but it was decided to focus on those that showed the highest expression variation between mutant and normal plants. The follow-up expression analysis was also narrowed to genes with the greatest expression differences and/or those that fall into defined functional categories. More than one-third of the differentially expressed genes are involved in carbohydrate metabolism, and four out of five of these are up-regulated in id1 mutant immature leaves (Table 1). A microarray profiling study of expression differences in vegetative tissues of Arabidopsis flowering time mutants also found a significant proportion of differentially expressed genes that are involved in metabolism (Wilson et al., 2005). Notable metabolism-related genes found in this study include genes encoding MDH and NADP-ME. MDH transcripts, which are not present in immature leaves of normal plants, are expressed at high levels in mutant immature leaves (Fig. 2B). MDH enzymes have diverse roles in plants, ranging from generating ATP in the citric acid cycle to converting oxaloacetate to malate in mesophyll cells of maize and sorghum. The tree was constructed in MEGA 3.1 (Kumar et al., 2004) based on a BioEdit 7.0 alignment (Hall, 1999). Five hundred bootstraps, pair-wise deletion of gaps, and a Poisson correction model were applied to generate the tree. An Arabidopsis thaliana glycosyl hydrolase family 1 protein (At5g44640) was used as an outgroup; At gluc.

*Molecular phenotype of indeterminate1 maize* 3689

Molecular phenotype of indeterminate1 maize

Molecular phenotype of indeterminate1 maize

Molecular phenotype of indeterminate1 maize

Molecular phenotype of indeterminate1 maize
whether loss of
and Colasanti, 2007), it will be interesting to determine
gene product do not fluctuate in a circadian pattern (Wong
needs to be examined. Although the
ID1
mutants, but whether there is a functional connection
between the
CHY RING zinc finger gene was found in
flower early in both LDs and SDs. Reduced transcription
showed that two of these genes, CSL and one of the CHY/
photosynthetic genes described above, RT-PCR analysis
showed that transcription of all of these genes is signifi-
cantly down-regulated in id1 immature leaves. Unlike the
photogenes described above, RT-PCR analysis
showed that two of these genes, CSL and one of the CHY/
RING finger genes (Zm059623), persist at lowered expres-
sion levels in mature green leaves. These genes have not
been characterized in maize and no clear functional
 equivalents in other plants have been reported. A protein
with similarity to the deduced maize CHY/RING finger protein described here is PEX10, an Arabidopsis peroxi-
somal H3HC4 RING finger protein that was recently
implicated in protein interaction required for attachment of
peroxisomes to chloroplasts and movement of metabolites
to and fro between peroxisomes and chloroplasts. Dysfunctional
pex10 mutants show abnormal respiration and decreased
quantum yield (Schumann et al., 2007).

A possible connection to flowering time regulation
could be made from a recent study of a RING-domain
zinc finger protein, RF12 (RED and FAR RED INSENSI-
TIVE2, At2g47700), which has a role in flowering time
control in Arabidopsis (Chen and Ni, 2006). RF12 was
reported to regulate the photoperiodic floral induction
pathway in Arabidopsis by fine-tuning the circadian
output received from phytochrome B and suppressing the
expression of CONSTANS (CO). As a result, rf12 mutants
flower early in both LDs and SDs. Reduced transcription
of the CHY RING zinc finger gene was found in id1
mutants, but whether there is a functional connection
between the Arabidopsis RF12 gene and flowering time
needs to be examined. Although the ID1 transcript and
gene product do not fluctuate in a circadian pattern (Wong
and Colasanti, 2007), it will be interesting to determine
whether loss of ID1 gene function can affect the circadian
rhythmicity of other genes.

The CSL-type of zinc finger protein contains a signature
motif of four conserved cysteine residues that bind a single
Zn2+ ion. The best studied genes of this class encode the
conserved eukaryotic mammalian DPH3 and DPH4
proteins, which are involved in the biosynthesis of
thiamine (Liu et al., 2006). The function of the CSL
motif is not known, but recent structural and comparative
studies have demonstrated that it may define a new family
of zinc ribbon proteins that can bind a single zinc ion (Sun
et al., 2005). Since zinc-binding proteins are known to
carry out a wide range of functions, it is difficult to
discern the roles of the zinc finger-encoding genes that are
down-regulated in id1 mutants based on sequence alone.
Furthermore, since their expression remains low in mature
leaves of id1 mutants, it is unlikely that they are direct
targets of the ID1 transcription factor, because ID1 is not
expressed in this tissue.

Zmdhr β-glucosidase gene expression is absent in id1
mutants

A striking finding in the microarray analysis is that three
related β-glucosidase genes are highly down-regulated in the
id1 mutant plants. One other β-glucosidase, Zmglu2,
was also shown to be down-regulated on the array, but
northern analysis and RT-PCR showed that this difference
was not so prominent and may be due to cross-
hybridization to highly similar gene family members.
Nevertheless, significant expression differences were
found for Zmdhr genes, suggesting that loss of ID1
function can affect a family of related genes. A particu-
larly striking finding is that the three Zmdhr genes have an
expression pattern that mimics that of ID1. This suggests
that ID1 may directly control the expression of these
genes. Genomic sequences for regions upstream of these
genes were not found in current databases, but once they
become available it will be possible to check for the
presence of the ID1-binding motifs (Kozaki et al., 2004).
Mutations in the Zmdhr genes identified here, or indeed
for any other maize β-glucosidase genes, have not been
described. Maize lines initially reported as ‘null’ for
β-glucosidase activity were later found to be caused by the
presence of a β-glucosidase-aggregating factor (BGAF)
(Esen and Blanchard, 2000). BGAF binds Zmglu1 and
Zmglu2 and causes them to aggregate in large insoluble
complexes, thereby rendering enzyme activity undetect-
able on in vitro assays. Therefore, the phenotypic
consequences of loss of function for maize β-glucosidase
genes, if any, have yet to be assessed. At least 34 members
of glycosyl hydrolase family 1 β-glucosidase genes have
been identified in rice (Opassiri et al., 2006); therefore, it
is likely that at least equal numbers of this class of genes
are present in maize. The closest Arabidopsis orthologues
of predicted maize β-glucosidase proteins and the Sbdhr1
protein all position within a single putative glycohydro-
lase subfamily sharing a close relationship in terms of
their biochemical function (Xu et al., 2004). Furthermore,
analysis with intracellular localization prediction software
WoLF PSORT (Horton et al., 2006) indicates that the
identified maize β-glucosidase proteins are likely to be
targeted to the chloroplast (data not shown). Therefore,
the β-glucosidases may also have a connection with
a possible alteration of photosynthetic potential in id1
mutants. However, the finding that the Zmdhr genes, like

Downloaded from http://jxb.oxfordjournals.org at University of Guelph on 2 April 2009
ID1, are not expressed in mature green leaves suggests that they might be associated with another type of plastid in developing leaves.

There are several scenarios with respect to a possible role for β-glucosidases in the ID1 floral induction pathway. The glycolytic hydrolases of the β-glucosidase class have been shown to have a variety of functions, such as hormone activation, cell wall synthesis, stress tolerance, and pathogen defence (Esen, 1993). Specificity for the aglycone portion of β-glucosidase substrates is quite broad. Non-cyanogenic β-glucosidase substrates such as Zmglu1 are able to catalyse the hydrolysis of a wide spectrum of natural and artificial substrates in addition to their natural substrate, the glucose-conjugated form of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin (DIMBOAGlc). Zmglu1 activity releases the DIMBOA aglycone, which is a factor involved in general defence against pathogens (Sicker et al., 2000). On the other hand, cyanogenic β-glucosidases, like the sorghum dhurrinases, recognize and cleave only dhurrin, their natural substrate. Dhurrin cleavage leads to the release of an activated toxic aglycone such as cyanide (HCN), which is also involved in plant defence against pathogens and herbivores (Cicek and Esen, 1998).

By creating reciprocal chimeras, Cicek et al. (2000) established that substitution of 487-NNNTRYMKE or 487-NNNCTRYMKE in Zmglu1 with the Sbdhr1 477-ENGCERTMKR and 481-SSGTTERF, respectively, resulted in chimeric Zmglu1 with an acquired ability to hydrolyse dhurrin. Sequence analysis of the three Zmdhr β-glucosidases revealed an overall higher degree of similarity to Sbdhr within the corresponding C-terminal regions, although none matches Zmglu1 or Sbdhr1 exactly. However, none of the Zmdhr genes identified here encodes the combination of key amino acid residues required for dhurrin cleavage, S-481, S-482, and R-487 (arrowheads, Fig. 4A).

Possible substrates for some or all of the Zmdhr proteins could be as diverse as cell wall components, polysaccharides, substances involved in defence, or glucose-conjugated plant hormones. Biochemical studies showed that the maize Zmglu1 gene activates hormone conjugates of cytokinin-O- and kinetin-N3-glucosides (Brzobohaty et al., 1993). More recently the Arabidopsis AtBG1 β-glucosidase was shown to have an important role in removing glucose conjugated to abscisic acid (ABA), thereby providing a mechanism for rapid ABA activation in response to environmental conditions (Lee et al., 2006). It is intriguing to speculate that a potential role for the Zmdhr proteins could be activation of glucose-conjugated compounds in developing leaves that can then migrate to the SAM to promote flowering. Basic phytohormones have been suggested to act as flower-inducing signals in some plants. In rye grass, Lolium temulentum, there is compelling evidence that gibberellic acid can act as a mobile florigenic signal (King and Evans, 2003; King et al., 2006). Although hormones have been proposed to be components of transmissible florigenic signals (Bernier and Perilleux, 2005), there is no evidence for this in maize. At this point, given the broad range of functions associated with β-glucosidase enzymes, it is not possible to ascribe a particular function to the Zmdhr genes based on sequence similarity, especially based on β-glucosidase proteins from divergent species (Henriatat and Davies, 2000). Biochemical analysis and metabolic profiling could provide more evidence for whether the novel Zmdhr proteins identified here catalyse the release of an active substance with a role in transmitting florigenic signals to the shoot apex.

Comparison of expression in florally induced teosinte and temperate maize

The photoperiod inducibility of teosinte was exploited to examine whether expression of any of the genes analysed here is different in induced versus uninduced plants. Overall, it was found that ID1, along with many of the other genes tested, showed little difference in transcript levels in immature leaves of SD- versus LD-grown teosinte plants (Figs 2B and 3B). However, notable exceptions include both zinc finger-encoding genes, CHY and CSL, which are up-regulated in florally competent ID1+ maize relative to idl mutants, and are also expressed at higher levels in flurally induced SD teosinte. Similarly, Zmdhr1 and Zmdhr2 transcripts are slightly, but consistently, higher in immature leaves of flurally induced SD teosinte when compared with LD plants. Because of the nature of photoperiod inducibility experiments it is possible that some of the observed expression differences in teosinte are due to total light integral differences between SD and LD treatments rather than effects related to flowering. Several of the genes tested, in fact, show the opposite to the expected pattern of expression differences in teosinte immature leaves. Notably, the IAA β-glucosyltransferase gene mRNA is detected at slightly lower levels in immature leaves of wild-type plants, but at elevated levels in flurally induced teosinte immature leaves. Interestingly, the Zmdhr3 gene is slightly up-regulated in uninduced teosinte, while its expression is virtually undetectable in idl mutants.

One possible reason that little concordance is seen between temperate maize and teosinte expression profiles might be because, in the case of photoperiod-induced teosinte, expression differences related to floral inductive signals are likely to be limited to mature green leaves. Physiological studies largely point to mature leaves as the source of florigenic signals (Imaizumi and Kay, 2006). The recent report of delayed flowering in maize phyB2/phyB1 photoreceptor mutants suggests that a latent, photoperiod-regulated floral induction pathway persists in temperate maize (Sheehan et al., 2007). The evolution of day-neutral maize from obligate SD photoperiod-requiring
progenitors was facilitated by ancient farmers as they migrated to higher latitudes. Perhaps during the rapid evolution of day-neutral maize the pre-existing SD-inducible pathway was superseded by a novel ID1-regulated autonomous floral induction pathway that is unique to temperate maize. Future profiling studies comparing expression differences between induced and uninduced teosinte mature leaves may reveal other genes that are associated with flowering. These can then be compared with the candidates described here to determine where these floral regulatory pathways intersect.

Transcriptome profiling reveals a diverse collection of genes that may be connected to floral induction

From this first comparative transcriptome profiling study between non-flowering id1 mutants and normal flowering maize plants, a relatively small number of genes that encode proteins of diverse functions have been identified. Many of the genes examined have apparent roles in plant metabolism in general, with specific connections to photosynthesis and carbon assimilation. Noticeably absent from this panoply of genes are potential Arabidopsis and rice orthologues that may have a role in regulating leaf-generated signals. In particular, no maize CO-related or FT-like genes were identified. This may be a limitation of the microarray that was used, i.e. although the 82 K array embodies a significant portion of the maize genome, rare regulatory genes, such as transcription factors, may be missing, be present as highly similar gene families that could obscure their detection, or be below the level of detection. At least 10 CO-related genes and several FT/TFL family member genes are present as probe sets on the 82 K microarray, but none showed significant differential expression between wild-type and id1 mutants (data not shown). It is conceivable that ID1 targets could have a role in facilitating the movement of a maize FT florigenic protein or other leaf-derived signalling molecules, and therefore loss of ID1 activity might not affect expression of these genes. However, whether any of the maize FT-like genes represent functional orthologues needs to be established. Another possibility is that ID1 regulates a pathway that is completely divergent from the CO/FT regulatory module described for other species (Corbesier et al., 2007; Tamaki et al., 2007). Certainly the complexity and importance of flowering in higher plants has left open the possibility that the floral transition can integrate signals from other internal or environmental stimuli. Many of the genes described in this study point to a link between plant metabolism and flowering time. Whether some or any of these genes have a direct role in flowering awaits functional analysis.

Supplementary material

Supplementary material available at JXB online includes relative fluorescence levels for all genes with 2-fold or greater difference (Fig. S1), and gels showing amplification products of the 13 genes analysed here by RT-PCR and genomic PCR with intron-spanning primers (Fig. S2). Table S1 gives sequences of primers used to analyse the 13 maize genes.

Acknowledgements

The authors thank Christina Shenton from Syngenta for conducting microarray experiments, Jing Zhang (Guelph Microarray Facility) for help with GeneSpring software, Michael Mucci (Guelph Phytooton) for plant care, Steven Rothstein and Mimi Tamimoto for useful discussions, and John Doebley (University of Wisconsin) for providing teosinte seeds. Research in JC’s lab is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC Discovery), the Canadian Foundation for Innovation, the Ontario Ministry of Agriculture and Food, and the Ontario Innovation Trust. VC is a recipient of an Ontario Graduate Scholarship award.

References


Cicek M, Blanchard D, Bevan DR, Essen A. 2000. The aglycone specificity-determining sites are different in 2, 4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (maize beta-glucosidase) and dhurrinase (Sorghum beta-glucosidase). Journal of Biological Chemistry 88, 20002–20011.


