Microarray analysis of vegetative phase change in maize

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Summary
Vegetative phase change is the developmental transition from the juvenile phase to the adult phase in which a plant becomes competent for sexual reproduction. The gain of ability to flower is often accompanied by changes in patterns of differentiation in newly forming vegetative organs. In maize, juvenile leaves differ from adult leaves in morphology, anatomy and cell wall composition. Whereas the normal sequence of juvenile followed by adult is repeated with every sexual generation, this sequence can be altered in maize by the isolation and culture of the shoot apex from an adult phase plant: an ‘adult’ meristem so treated reverts to forming juvenile vegetative organs. To begin to unravel the as-yet poorly understood molecular mechanisms underlying phase change in maize, we compared gene expression in two juvenile sample types, leaf 4 and culture-derived leaves 3 or 4, with an adult sample type (leaf 9) using cDNA microarrays. All samples were leaf primordia at plastochron 6. A gene was scored as ‘phase induced’ if it was up- or downregulated in both juvenile sample types, compared with the adult sample type, with at least a twofold change in gene expression at a $P$-value of $\leq0.005$. Some 221 expressed sequence tags (ESTs) were upregulated in juveniles, and 28 ESTs were upregulated in adults. The largest class of juvenile-induced genes was comprised of those involved in photosynthesis, suggesting that maize plants are primed for energy production early in vegetative growth by the developmental induction of photosynthetic genes.

Keywords: phase change, leaf development, microarray, maize, plastochron 6, heteroblastic.

Introduction
Like all multicellular organisms, angiosperms pass through a series of developmental states to complete their life cycles. A particularly prominent transition is that from vegetative growth to flowering, when sexual reproduction occurs (Simpson et al., 1999; Steeves and Sussex, 1988). For this transition to occur, the plant must be an adult. That is, a transition in vegetative growth from juvenile to adult precedes the transition from vegetative to reproductive development (Poethig, 1990). This transition between the juvenile phase and the adult phase is called vegetative phase change (Brink, 1962; referred to as phase change hereafter). In many species, vegetative traits change in coordination with the gain of the ability to flower – adult organs are marked by a pattern of differentiation that is distinct from that of juvenile organs (Hackett, 1985; Kerstetter and Poethig, 1998). Understanding the mechanisms that coordinate the regulation of adult vegetative differentiation with the gain of floral competence remains a fundamental challenge in plant biology.

In maize, the shoot meristem initiates a genotype-specified number of leaves prior to producing a terminal inflorescence: the tassel. In most genetic backgrounds, the first four or five leaves are juvenile (Freeling, 1992). Phase change occurs during the differentiation of the next two or three leaves formed, which are referred to as transition leaves. Transition leaves are mosaics of both juvenile and adult tissue (Bongard-Pierce et al., 1996). Leaves initiated after phase change differentiate entirely as adult leaves (Poethig, 1990).

Heteroblastic variation between the juvenile and adult phases in maize is most easily observed through morphological and histochemical differences of the epidermis of a
fully expanded leaf blade (Bongard-Pierce et al., 1996; Evans et al., 1994; Freeling and Lane, 1994; Moose and Sisco, 1994; Poethig, 1990). The surface of juvenile leaves lacks trichomes (macrohairs), and has a dull blue-green appearance caused by the presence of epicuticular wax. Trichomes are a prominent feature of the surface of adult leaves, which have a glossy appearance because epicuticular wax is substantially reduced, as compared with seedling leaves. Anatomically, juvenile leaves are characterized by weakly crenulated walls of epidermal cells in peridermal view, which appear violet when stained with Toluidine Blue-O. In cross section, epidermal cells of juvenile leaves are round, in contrast to cuboidal in the adult leaves. Epidermal cell walls in adult leaves are characterized by their strong crenulation in peridermal view, and their aquamarine staining with Toluidine Blue-O, the latter indicating higher levels of lignin compared with juvenile leaves. Juvenile and adult leaf blades also differ with respect to other components of the cell wall, with juvenile blades having higher total uronosyl and glucose, but lower levels of neutral sugars, xylose, ferulate and ferulate dimers (Abedon et al., 2006).

Several mutations have been described in maize that hasten or delay the appearance of adult-phase traits. Recessive, loss-of-function mutations in the glossy15 (gl15) gene cause the precocious expression of epidermal traits associated with the adult phase (Evans et al., 1994; Moose and Sisco, 1994). The gl15 gene encodes a protein that contains a domain with significant homology to the DNA-binding domain of APETALOA2 (AP2; Moose and Sisco, 1996), which is an Arabidopsis transcription factor. As is the case for AP2 (Aukerman and Sakai, 2003), gl15 is a target of the microRNA miR172 (Lauter et al., 2005). The loss-of-function maize early phase change (epc) mutation dramatically reduces the number of juvenile and transition leaves, thereby causing the precocious expression of adult traits and early flowering (Vega et al., 2002). In contrast, Teopod2 (Tp2) and Corngrass1 (Cg1) are dominant, gain-of-function mutations that extend the expression of a suite of juvenile traits into the adult phase (Poethig, 1988). The recent cloning of Cg1 by Chuck et al. (2007) revealed that it encodes two tandemly arrayed miR156 genes. Thirteen putative SQUAMOSA-PROMOTER-BINDING-PROTEIN-LIKE (SPL) genes in maize, which have target sites for miR156, have been identified (Chuck et al., 2007). Loss-of-function mutations that decrease levels of bioactive gibberellic acid (GA), such as dwarf1 (d1), also prolong the juvenile phase, and, in addition, delay the onset of the adult phase and flowering (Evans and Poethig, 1995).

Phase change in maize can also be altered experimentally by culturing the shoot apex (Irish and Nelson, 1988). Culture-derived plants recapitulate normal shoot development, producing juvenile, transition and adult nodes that are similar in number to, and are qualitatively indistinguishable from, those observed for seed-derived plants. Regardless of whether the shoot apex was derived from a juvenile or an adult plant, the first formed leaves have juvenile characters (Irish and Karlen, 1998; Orkwiszewski and Poethig, 2000): i.e. the morphogenetic program of the shoot is ‘reset’ upon excision and culture. Thus, the relationship between the age of the meristem and its developmental program are uncoupled through culture-induced rejuvenation (Irish and Karlen, 1998; Irish and McMurray, 2006).

Here, we report the identification of genes differentially expressed in a phase-induced manner in leaf primordia at plastochron 6 (P6) in maize by the microarray analysis of juvenile leaf 4, adult leaf 9 and juvenile leaves 3 or 4 from culture-rejuvenated plants. We identified 221 and 28 genes that were upregulated in the juvenile and adult phases, respectively. The largest class of juvenile-induced genes was comprised of those involved in photosynthesis. Whereas photosynthetic genes have been long known to be induced by light, this observation suggests that in order to prime maize plants for energy production early in vegetative growth, the induction of photosynthetic genes relies on developmental cues.

### Results

To identify genes that may be responsible for the different patterns of phase-specific differentiation, we compared gene expression profiles of juvenile leaf 4 (L4) and adult leaf 9 (L9) primordia. As a control for the juvenile state, we included in our comparison a third sample, culture-rejuvenated leaf 3 or 4 (RL3/4) primordia. In this way we could eliminate genes that were up- or downregulated early in seedling development, such as those that might be associated with germination, for example, but that would not be truly associated with the juvenile phase. Six independent biological replicates for L4, L9 and RL3/4 were harvested from primordia at P6. RNA extracted from these samples was reverse transcribed, and each target cDNA population was labeled with either a Cy3 or Cy5 florescent dye, in accordance with the experimental design (see Experimental procedures). Dye-labeled RNA populations from the individual sample replicates were co-hybridized to cDNA microarrays that contained 7886 informative elements. Each hybridized microarray was scanned six times, and each time the laser power and photomultiplier tube gain for each dye channel was increased.

Identification of candidate phase-related genes

Differentially expressed expressed sequence tags (ESTs) were identified using two criteria: a P-value of ≤0.005, and an expression ratio that was greater than or equal to 2.0. Estimated false discovery rates (FDR) of <2.3% were calculated for those ESTs with P-values ≤0.005 (Figures 1 and S1).
Points in Figure 1 to the right of the vertical line represent ESTs for which expression differences were highly significant ($P \leq 0.005$). Points above the upper or below the lower horizontal lines in each plot represent ESTs that show a twofold or greater difference in expression. Hundreds of ESTs showed significant differences in expression in the three pairwise comparisons (L4 versus L9, L9 versus RL3/4 and RL3/4 versus L4; Figure 1). Further filtering of the data sets was accomplished by applying a third criterion: an EST was classified as phase-induced only if it was up- or down-regulated in both L4 and RL3/4, as compared with L9. These criteria yielded 221 ESTs that were upregulated in juvenile samples, and 28 ESTs that were upregulated in adult samples (Figure 2; Tables 1, 2 and S1).

**Expression level comparisons between the seed-derived juvenile state and the culture-rejuvenated state**

Although we defined a gene as being phase-induced if it showed at least a twofold difference in expression between the two phases, no other constraints were applied to the data set: i.e. we did not specify how much greater than twofold the upregulation of the expression should be. To determine the extent to which gene expression mirrors morphological similarity, we compared the expression profiles of L4 versus

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Table 1 Selected expressed sequence tags (ESTs) that were upregulated in both juvenile leaf 4 and rejuvenated leaves 3/4 compared with adult leaf 9

<table>
<thead>
<tr>
<th>EST GenBank AC</th>
<th>Top BLASTX annotation</th>
<th>Low-scan set&lt;sup&gt;c&lt;/sup&gt;</th>
<th>High-scan set&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L4 versus L9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>RL3/4 versus L9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-value&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Ratio&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM339253</td>
<td>Immunophilin, putative/FKBP-type pept idyl-prolyl cis-trans isomerase, putative [NP_568067.1 (172; 2e–41; A. thaliana)]</td>
<td>2.06E–05</td>
<td>132.00</td>
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<tr>
<td>BM080125</td>
<td>Photosystem II subunit PsbS1 [NP_00105228.1 (73.9; 3e–12; Z. mays)]</td>
<td>2.36E–04</td>
<td>100.97</td>
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<tr>
<td>BM074536</td>
<td>NADP-malic enzyme [AAP33011.1 (150; 7e–35; Z. mays)]</td>
<td>1.15E–03</td>
<td>55.49</td>
</tr>
<tr>
<td>BG840776</td>
<td>One helix protein [AAP33011.1 (115; 2e–24; D. antarctica)]</td>
<td>2.52E–04</td>
<td>43.62</td>
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<tr>
<td>BM267937</td>
<td>Photosystem-I reaction center subunit-II, chloroplast precursor (Photosystem-I 20-kDa subunit) (PSI-D) [P36213.1 (203; 4e–51; H. vulgare)]</td>
<td>5.67E–04</td>
<td>37.91</td>
</tr>
<tr>
<td>DV550281</td>
<td>RISBZ4 [BAD26199.1 (157; 8e–37; O. sativa)]</td>
<td>5.94E–04</td>
<td>37.56</td>
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<td>BG840818</td>
<td>Putative protodermal factor [AAP33011.1 (93.2; 1e–17; O. sativa)]</td>
<td>1.26E–04</td>
<td>36.75</td>
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<tr>
<td>DV491668</td>
<td>Chlorophyll a/b binding protein 48, chloroplast precursor (LHCII type-I CAB-40) (LHCP) [Q00827.1 (490; 1e–136; Z. mays)]</td>
<td>2.47E–04</td>
<td>18.15</td>
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<td>DV494632</td>
<td>Homeobox protein HD1, putative, expressed [ABF93721.1 (114; 2e–24; O. sativa)]</td>
<td>1.85E–03</td>
<td>15.49</td>
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<tr>
<td>BM074151</td>
<td>ATNSI (NUCLEAR SHUTTLE INTERACTING); N-acetyltransferase [NP_973950.1 (88.2; 2e–16; A. thaliana)]</td>
<td>3.09E–04</td>
<td>14.03</td>
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<td>BM341648</td>
<td>Putative very-long-chain fatty acid condensing enzyme CUT1 [BAD15940.1 (175; 1e–42; O. sativa)]</td>
<td>9.98E–04</td>
<td>13.77</td>
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<td>BM334653</td>
<td>Ribulose bisphosphate carboxylase small subunitI [NP_001105294.1 (268; 3e–69; Z. mays)]</td>
<td>5.29E–04</td>
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<td>BM073471</td>
<td>Elongation factor family protein [NP_851035.1 (91.3; 5e–17; A. thaliana)]</td>
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<td>10.97</td>
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<tr>
<td>BM350700</td>
<td>F-box family protein, putative, expressed [ABF939658 (101; 1e–19; O. sativa)]</td>
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<td>– –</td>
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<td>BG842033</td>
<td>Catalase isozyme 3 [P18123.1 (201; 2e–50; Z. mays)]</td>
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<tr>
<td>BG840924</td>
<td>ABC transporter family protein [NP_175837.2 (73.6; 6e–12; A. thaliana)]</td>
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<td>7.80</td>
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<tr>
<td>DV498355</td>
<td>Putative photosystem-I reaction center subunit IV [BAC84088.1 (144; 2e–33; O. sativa)]</td>
<td>1.59E–03</td>
<td>7.10</td>
</tr>
<tr>
<td>BG841780</td>
<td>Polyamine oxidase [CAC04002.1 (280; 6e–74; Z. mays)]</td>
<td>3.18E–03</td>
<td>6.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative P-value for L4 versus L9 is relative to the proportion of low-scan set hits; and P-value for RL3/4 versus L9 is relative to the proportion of high-scan set hits. 
<sup>b</sup> BLASTX annotations and GenBank Accession numbers in square brackets. 
<sup>c</sup> Low-scan set vs high-scan set. 
<sup>d</sup> Adult leaf 9.

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L9 with those of RL3/4 versus L9. The mean ratio of the L4 versus L9 comparisons with the RL3/4 versus L9 was 0.91 ± 0.03 (±SEM) (Figure 3a). This suggests that genes that are upregulated in juvenile and culture-rejuvenated samples are expressed at similar levels. A similarly high degree of correlation was calculated for the L9 versus L4 and L9 versus RL3/4 comparisons (Figure 3b), which had a mean ratio of 0.97 ± 0.06.

Functional classification of candidate juvenile and adult upregulated ESTs

The annotation of the juvenile- and adult-induced ESTs was determined by using BLASTX, which compares conceptual translation products with a protein database in GenBank (Tables 1, 2 and S1). Information from the Gene Expression and Visualization Application (GENEVA; http://...
Table 2: Expressed sequence tags (ESTs) that are upregulated in adult leaf 9 compared with both juvenile leaf 4 and rejuvenated leaves 3/4

<table>
<thead>
<tr>
<th>EST GenBank AC</th>
<th>Top BLASTX annotation [GenBank AC] (score; e-value; species)b</th>
<th>Low-scan setc</th>
<th>High-scan setc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L9 versus L4d</td>
<td>L9 versus RL3/4d</td>
<td>L9 versus L4d</td>
</tr>
<tr>
<td></td>
<td>P-valuee</td>
<td>Ratiof</td>
<td>P-valuee</td>
</tr>
<tr>
<td>DV943135</td>
<td>Hypothetical protein OsJ_000126 [EAZ10309.1 (101; 8e–21; O. sativa)]</td>
<td>7.19E–05</td>
<td>36.80</td>
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<tr>
<td>DV943306</td>
<td>605 ribosomal protein L27 (RPL27C) [NP_193236.1 (175; 8e–42; A. thaliana)]</td>
<td>3.75E–06</td>
<td>30.39</td>
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<tr>
<td>AI692021</td>
<td>Putative somatic embryogenesis protein kinase 1 [BAD68873.1 (119; 1e–25; O. sativa)]</td>
<td>1.20E–03</td>
<td>22.48</td>
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<tr>
<td>DV493121</td>
<td>Os05g03566000 [NP_001055294.1 (136; 2e–29; O. sativa)]</td>
<td>4.52E–04</td>
<td>12.95</td>
</tr>
<tr>
<td>AI734769</td>
<td>Class III HD-Zip protein 4, putative, expressed [ABF97 828.1 (211; 1e–53; O. sativa)]</td>
<td>5.91E–04</td>
<td>11.99</td>
</tr>
<tr>
<td>BM078628</td>
<td>Putative growth-regulating factor 13 [NP_00106644.1 (156; 7e–96; Z. mays)]</td>
<td>2.16E–03</td>
<td>9.25</td>
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<tr>
<td>BM078110</td>
<td>WW domain containing protein, expressed [ABA00833.2 (135; 8e–30; O. sativa)]</td>
<td>1.06E–03</td>
<td>8.58</td>
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<tr>
<td>DV489742</td>
<td>SEC14 cytosolic factor (secretion factor 14) family protein [CAJ76360.1 (155; 1e–36; B. sylvaticum)]</td>
<td>2.82E–05</td>
<td>6.79</td>
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<td>BM072793</td>
<td>n.s.</td>
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<td>–</td>
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<tr>
<td>DY576313</td>
<td>Protein kinase family protein [NP_973956.1 (180; 1e–57; A. thaliana)]</td>
<td>2.04E–04</td>
<td>5.50</td>
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<tr>
<td>DV493254</td>
<td>Protein kinase family protein [NP_666630.1 (135; 7e–29; A. thaliana)]</td>
<td>1.99E–04</td>
<td>5.45</td>
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<td>DV943127</td>
<td>Helix-loop-helix DNA-binding [ABD23380.2 (78.2; 2e–12; M. truncatula)]</td>
<td>2.12E–03</td>
<td>5.29</td>
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<td>DV943234</td>
<td>n.s.</td>
<td>2.62E–03</td>
<td>4.30</td>
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<td>AI739394</td>
<td>Putative lateral organ boundaries (LOB) domain family [AAT42184.1 (75.1; 5e–11; Z. mays)]</td>
<td>6.86E–05</td>
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<td>BM333901</td>
<td>n.s.</td>
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<td>–</td>
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<td>DV493108</td>
<td>Protein kinase family protein [NP_973956.1 (180; 1e–57; A. thaliana)]</td>
<td>3.50E–03</td>
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<td>DV494242</td>
<td>Putative growth-regulating factor 13 [NP_00106644.1 (156; 7e–96; Z. mays)]</td>
<td>3.75E–03</td>
<td>2.97</td>
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<tr>
<td>DV495600</td>
<td>Squamosa promoter-binding-like protein 13 [OBA4616.1 (82; 9e–14; O. sativa)]</td>
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<td>DV942270</td>
<td>n.s.</td>
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<td>–</td>
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<td>DV492592</td>
<td>Hypothetical protein OsJ_017000 [EAZ33517.1 (208; 2e–52; O. sativa)]</td>
<td>1.38E–04</td>
<td>2.83</td>
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<td>DV942189</td>
<td>Hypothetical protein Os1_06629 [EAY95396.1 (150; 7e–25; O. sativa)]</td>
<td>1.51E–03</td>
<td>2.79</td>
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<tr>
<td>DV942476</td>
<td>Putative FH protein NH2 [BAB86673.1 (189; 2e–46; O. sativa)]</td>
<td>5.93E–04</td>
<td>2.67</td>
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<tr>
<td>BM075057</td>
<td>Cyclo-dependkinases regulatory subunit [AAS13367.1 (144; 3e–33; G. maxl)]</td>
<td>2.35E–03</td>
<td>2.52</td>
</tr>
<tr>
<td>BM073426</td>
<td>OSJnBa041M21.1 [CAD40443.2 (199; 1e–49; O. sativa)]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DV942221</td>
<td>Hypothetical protein OsJ_002940 [EAZ31131.5 (85.9; 5e–27; O. sativa)]</td>
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<tr>
<td>BG482886</td>
<td>LIM transcription factor homolog [NP001104937.1 (239; 8e–71; Z. mays)]</td>
<td>3.88E–03</td>
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<td>DV489762</td>
<td>Unknown protein [NP_564611.1 (90.1; 7e–17; A. thaliana)]</td>
<td>2.22E–03</td>
<td>2.11</td>
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<tr>
<td>BM072028</td>
<td>Inorganic pyrophosphatase [NP_001104889.1 (171; 1e–56; Z. mays)]</td>
<td>4.22E–03</td>
<td>2.05</td>
</tr>
</tbody>
</table>

**Notes:**
- ESTs presented in this table are upregulated by at least twofold in leaf 9 compared with both leaf 4 and rejuvenated leaves 3/4 at P ≤ 0.005.
- BLASTX screen of individual EST against the NCBI nr (February 24, 2008); n.s., no significant BLAST hit (e-value cut-off of 1e–10).
- See Experimental procedures; Data acquisition.
- Individual P-values obtained from the normalized log-scale signal intensity with the mixed linear model.
- Antilog of estimated log scale expression differences.
- BLASTX screen of individual MAGIv4 (e-value cut-off e–100) against the NCBI nr (February 24, 2008).
- ESTs in boldface are upregulated in both leaf 9 and rejuvenated leaves 3/4.
- ESTs in italics are upregulated in leaf 9 compared with leaf 4.
- ESTs in plain text are not upregulated in any condition.

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and The Arabidopsis Information Resource (http://www.arabidopsis.org) was used to group the ESTs identified in this study into 14 functional categories (Figure 4). Non-annotated genes occupied the largest groups within each data set: 27.1% (60/221) and 39.3% (11/28) for juvenile and adult data sets, respectively. A large fraction of annotated ESTs that were upregulated in juvenile leaves are involved in photosynthesis. This class represents 32.2% (52/161) of the annotated juvenile upregulated ESTs, and includes ESTs that encode putative proteins of photosystems I and II, light harvesting complexes, electron transport and the Calvin cycle. Other prominent functional groups that were upregulated in juvenile leaf primordia include metabolism (28/161), translation (20/161) and transport (13/161). Two main functional groups were upregulated in the annotated adult-induced data set: transcription (8/17) and signal transduction (5/17). No homologs of juvenile-upregulated photosynthetic genes were found to be upregulated in adult tissues.

Confirmation of microarray data by semiquantitative RT-PCR and RNA blot analyses

To validate the results of the microarray experiments, the transcript levels of five ESTs upregulated in juvenile leaves, and three ESTs upregulated in adult leaves, were examined by semiquantitative reverse transcriptase (RT)-PCR and RNA blot analyses using total RNA prepared from independently isolated, P6-stage leaf primordia samples (Figure 5). Except for some quantitative variation in the relative expression levels, the results were consistent with the phase-induced patterns of EST expression from the microarray data. Two ESTs from each class of phase-induced ESTs were selected for further analysis, based on their annotation: the juvenile upregulated putative immunophilin/FKBP-type peptidyl-prolyl cis-trans isomerase
was, like leaves. Expression of the juvenile upregulated expression was not detected in transition (5–7) or adult (8–14) leaves. Transcript levels for the juvenile upregulated plant is accurately represented by the expression of others (ZmFkb and ZmSpl) could only be detected in one phase.

**Discussion**

A number of mutants have been identified that hasten or delay phase change in maize (Chuck *et al.*, 2007; Evans and Poethig, 1995, 1997; Evans *et al.*, 1994; Moose and Sisco, 1994; Poethig, 1988; Vega *et al.*, 2002). Phase change can also be manipulated in maize by use of shoot apex culture, in which the developmental program of an adult meristem is ‘reset’ back to the juvenile stage when it is excised and cultured (Irish and Karlen, 1998; Irish and Nelson, 1988). That is, the first leaves that an excised meristem initiates differentiate as juvenile leaves, even if the meristem came from an adult plant, such that the shoot recapitulates phase change. Mutant analyses and shoot apex culture experiments have contributed to our understanding of phase change in maize. However, the genes responsible for initiating the transition,
as well as those that respond to it, have not yet, for the most part, been identified.

Global level comparisons of gene expression confirm the juvenile phase is recapitulated by shoot apex culture

Juvenile leaves of culture-derived shoots are morphologically and histologically indistinguishable from juvenile leaves of seed-derived plants (Irish and Karlen, 1998; Orkiewizewski and Poethig, 2000). We compared two independent ‘juvenile’ samples and found that 66% (221/337) of the ESTs that were upregulated in the L4 versus L9 data set were also upregulated in the RL3/4 versus L9 data set (Figure 2a).

In addition, the expression values for the 221 upregulated and the 28 downregulated ESTs showed a strong correlation between the two data sets (Figure 3a,b). The fact that such a large proportion of ESTs from the two data sets are upregulated to similar levels provides support that shoot apex culture ‘resets’ the genetic networks that regulate juvenile development, and thus validates the experimental approach employed in this study.

Candidate annotated phase-induced ESTs are consistent with the biology of phase-specific differentiation

Several of the annotated phase-induced ESTs identified in this study complement what is already known about phase-specific differentiation in maize. The blade of juvenile leaves has a dull, blue-green appearance caused by the presence of epicuticular wax on the blade surface. Two juvenile upregulated ESTs are thought to function in epicuticular wax synthesis and epidermis-to-cuticle export of lipids. The putative very long chain fatty acid (VLCFA) condensing enzyme, CUT1 (BM341648), showed a 13.8-fold higher expression in L4 compared with L9 (Table 1). In Arabidopsis, CUT1 is expressed in the epidermis, and is required for the synthesis of VLCFA precursors (Millar et al., 1999). Suppression of CUT1 results in organs with a waxless
phenotype resembling other mutations, such as those in the *eceriferum* (cer) loci in Arabidopsis, and in the *glossy* loci in maize, that cause defects in epicuticular wax biosynthesis or accumulation (Millar et al., 1999). This analysis also revealed a fourfold upregulation of an EST annotated as a putative adenosine triphosphate binding cassette (ABC) transporter (DV491027) in L4 and RL3/4 (Table 1). The *CER5* gene in Arabidopsis encodes an ABC transporter required for wax export to the cuticle (Pighin et al., 2004). cer5 mutations result in glossy stems, indicating a reduction in cuticular wax (Pighin et al., 2004).

The juvenile upregulated data set is dominated by genes involved in photosynthesis

Some 30% of the annotated ESTs in the juvenile data set are involved in photosynthesis (Figure 4). This is true even though P6 primordia are pale yellow, are shaded by a whorl of expanded leaves and are thus unlikely to be undergoing photosynthesis. Langdale et al. (1988) demonstrated that genes involved in photosynthesis are expressed at the early stages of leaf development in maize, independent of light. For example, in an undifferentiated maize leaf blade, RuBPCase large- and small-subunit genes are expressed concurrently with provascular cell divisions, but prior to extensive vascular differentiation. This expression pattern occurs at a stage in leaf development when neither bundle sheath cells nor mesophyll cells have become morphologically distinct, and before chloroplasts can be distinguished by light microscopy (Langdale et al., 1988). The light-independent induction of photosynthesis genes has been documented in Arabidopsis through the expression profiling stages of embryogenesis (Spencer et al., 2007). During the transitions from globular, to heart, to torpedo stages of embryo development, genes involved in energy production comprise the largest functional group that is upregulated, with a strong bias towards the genes encoding components of the photosynthetic apparatus (Spencer et al., 2007).

An EST annotated as a putative Cab48, which showed an 18.2-fold higher expression in L4 than in L9 by microarray analysis, was chosen as a representative of the photosynthesis-related class of genes for examining the pattern of expression in the successive P6-staged leaves of wild-type plants. Developmental regulation of *Cab* genes and other photosynthetic genes has been reported in Arabidopsis (Brusslan and Tobin, 1992; Chory et al., 1991), *Glycine max* (Chang and Walling, 1992), *Pisum sativum* (He et al., 1994) and *Amaranthus hypochondriacus* (Ramsperger et al., 1996). Juvenile upregulated expression of *Cab* has been reported in English ivy (Woo et al., 1994). In wild-type maize plants, *Cab48* expression was confined to the primordia of the first five leaves, which, in the genetic background used throughout this study, represents the extent of the juvenile phase (Figure 6b,d). Whereas light is known to be required for the induction of expression of many genes involved in pigment biosynthesis and in the photosynthetic machinery, developmental cues are also thought to play some role in the regulation of photosynthetic genes. Such developmental regulation could be important for preparing the juvenile seedling for the rapid biogenesis of the photosynthetic apparatus, and thus, energy production, shortly after the emergence of the coleoptile during germination.

ZmFkp expression is regulated in an early juvenile-induced manner

The expression of a second juvenile upregulated EST, annotated as *ZmFkb*, was examined in successive leaves of wild-type plants. FK506-binding proteins (FKBPs), along with cyclophilins, belong to the immunophilin superfamily (Romano et al., 2005). Members of both the FKBPs and cyclophilin families function similarly to chaperones in assisting protein folding (Romano et al., 2005). FKBPs and cyclophilin proteins have an N-terminal peptidyl prolyl isomerase (PPIase) domain and three tetra-tricopeptide (TPR) domains located in the C terminus (Pratt et al., 2001; Romano et al., 2005). The PPIase activity isomerizes prolyl bonds from the cis to the trans configuration (Reimer and Fischer, 2002). Mutations in the Arabidopsis cyclophilin 40 ortholog, *sqn*, eliminate nearly all transition leaves, and dramatically accelerate phase change, indicating a role for the wild-type gene in maintaining the juvenile phase. The C-terminal TPR triplet of *SQN* is required for interacting with Hsp90 (Berardini et al., 2001). A putative *ZmFkb* was identified by microarray analysis as being highly upregulated in juvenile samples, with a 132-fold higher expression in L4 compared with L9 (Table 1). RNA blot analysis confirmed its juvenile phase expression, but with the highest levels in L1 and L2 (Figure 6a,c), and much lower levels in all successive leaves, rather than the dramatic difference in expression between L4 and L9, as indicated by the microarray analysis.

ZmSPL is regulated in a highly phase-induced manner

The largest classes of annotated adult upregulated ESTs are classified as functioning in signal transduction or transcription. We examined the expression profiles of putative *ZmSek* and *ZmSPL* genes, representing both of these functional categories, in successive P6-staged leaves from wild-type plants. In Arabidopsis, 11 of the 17 *SPL* genes—including *SPL3*, *SPL4* and *SPL5*—are targeted by *miR156* (Gandikota et al., 2007; Rhoades et al., 2002; Schwab et al., 2005), and in rice, 11 out of the 19 predicted *SPL* genes—including OsSPL13—have *miR156* target sites (Xie et al., 2006). *SPL* expression in Arabidopsis is antagonized by *miR156* in juvenile leaves, where *miR156* levels are at their highest (Wu and Poethig, 2006). Upon phase change, *miR156* levels decrease and *SPL3* mRNA levels
subsequently increase. In Arabidopsis, SPL3 regulates the expression of a subset of adult traits, such as abaxial trichome distribution and petiole length (Wu and Poethig, 2006). Few members of the SPL family have been characterized in maize. Mutant analyses of the maize SPL genes liguleless1 (lg1) and teosinte glume architecture (tga) indicate that these genes are critical for leaf and glume development, respectively (Moreno et al., 1997; Wang et al., 2005). lg1 encodes a nuclear-localized protein with a squamosa promoter binding protein (SBP) domain (Moreno et al., 1997), and functions to promote epidermal cell fate with expression in leaf primordia at or prior to P6 (Moreno et al., 1997). Recently, it was found that CgTtl encodes an miR156 RNA (Chuck et al., 2007). Thirteen potential SPL genes, including tga, that are expected to be targets of CgTtl were identified (Chuck et al., 2007). We found that the level of ZmSpl mRNA increased in successive leaves, beginning with the early transition L5 in wild-type plants (Figure 6b,f). ZmSPL is a putative ortholog of OsSPL13 which groups with SPL3/4/5 of Arabidopsis in an unrooted tree based on the protein sequence of the SBP domain (Xie et al., 2006). The similarity among the expression profiles of ZmSpl in maize (this study) and SPL3/4/5 in Arabidopsis, which are targeted and cleaved by miR156 in a phase-induced manner (Wu and Poethig, 2006), suggests that these are regulated by similar mechanisms, and have conserved functions in establishing the adult phase in these two angiosperm species.

Experimental procedures

Plant material and plant growth conditions

The wild-type maize plants used throughout this study were F1 hybrids from a cross between W23 and stock 924A from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). The 924A stock carries white deficiency, and is of a genetic background that is one quarter M14/W23 and three quarters unique. This S8 W23 hybrid shows superior performance in shoot apex culture, compared with the W23 inbred line (EI, unpublished data), which was required for these experiments. In this line, L1–L4 differentiated entirely as juvenile leaves, whereas L5 and above differentiated entirely as adult leaves. The L5–L7 leaves were transition leaves, showing juvenile traits at the first-differentiating tip, and adult traits closer to the base of the leaf blade. Maize kernels were sown in Jiffy Plus potting mix (Jiffy Products of America Inc., http://www.jiffypot.com) in 8.5-cm-diameter plastic pots. Plants were grown in the greenhouse with a 16-h light/8-h dark photoperiod provided by 1000 W high-pressure sodium and metal halide lamps.

Shoot apex culture

Shoot apices used for shoot apex culture were harvested from W23/924A hybrid plants at approximately 3 weeks after planting, when they had initiated 12 leaves in total and showed five or six expanded leaves. Shoot apices consisting of the meristem plus the two newest leaf primordia were cultured. Rejuvenated L3 and L4 leaves thus correspond to the first two leaves formed by the meristem after its excision from the plant, and would have been L13 and L14 on the plant had the shoot apex not been excised. Shoot apex culture was performed as described previously (Irish and Nelson, 1988).

RNA isolation

Leaf primordia at P6 (i.e. the stage that a leaf reaches during the period in which five additional leaves have been initiated by the shoot meristem, which corresponds to a leaf length between 4 and 6 mm in the line used here) were collected for L4 and L9 from seed-derived plants, and L3 or L4 from culture-derived plants. Total RNA was extracted from samples consisting of approximately 24 leaf primordia using Trizol reagent (Invitrogen, http://www.invitrogen.com) following the manufacturer’s recommendations.

Experimental design and microarray procedures

For each of six biological replications, each of the three pairwise comparisons of P6-staged leaf primordia from L4, L9 and RL3/4 was made on one slide. With six biological replications and three slides per replication (L4 versus L9, L9 versus RL3/4 and RL3/4 versus L4), this replicated loop design used a total of 18 slides. To ensure dye balance, each of the 18 target samples was measured once with Cy3 labeling, and once with Cy5 labeling. Fluorescently labeled cDNA targets were prepared and hybridized to a 12 160-element cDNA microarray chip (Generation II, version B) according to the protocol available at http://schnablelab.plantgenomics.iastate.edu/resources/protocols. The microarray was generated at the Center for Plant Genomics at Iowa State University (http://www.plantgenomics.iastate.edu/maizechip; Nakazono et al., 2003).

Data acquisition

Microarray slides were scanned with a ScanArray 5000 (Hewlett Packard Inc., http://www.hp.com). A multiple scanning method similar to that described by Skibbe et al. (2006) was implemented. Fluorescent signal intensities were determined using Imagene 5.0 (Biodiscovery, http://www.biodiscovery.com). Scan pairs with Cy3 and Cy5 scan intensities of similar median values over all replicate slides constituted a scan set. Two scan sets (a low- and high-scan set) that had approximate median natural log signals of 6 and 7 prior to normalization were selected from all six scans of the 18 slides for analysis.

Data normalization

The Lowess normalization method (Dudoit et al., 2002) was applied to the log of the background-corrected raw signal intensity (signal intensity minus the median background intensity) to remove signal-intensity-dependent dye effects on each slide. Lowess normalization was performed separately for each slide to avoid the introduction of dependence among the independent biological replicates. The normalized data for each slide/dye combination were mean-centered (each individual value associated with a particular slide/dye combination minus the average value associated with the particular slide/dye combination), so that expression measures would be comparable across all slides. As a result, negative (positive) values were indicative of a particular transcript being expressed below (above) the average for a particular slide/dye combination.
Data analysis

Of the 12,160 spots contained on the Generation-II version-B cDNA array, 1520 'empty' or 'bad-PCR' spots were removed from the data set prior statistical analysis. A mixed linear model analysis (Wolfe et al., 2001) of the normalized log-scale signal intensities for each of the 10,640 spots on the array was performed to identify significant transcripts differentially represented among juvenile, adult and culture-rejuvenated leaves. The mixed linear model included fixed effects for leaf number/type (L4, L9 or RL3/4) and dye (Cy3 or Cy5), as well as random effects for day of hybridization, replication, slide and sample. As part of each mixed linear model analysis, a Student's t-test for differential gene expression was conducted for each pair-wise comparison of leaf number/type. The proportion of false-positive results among all genes with \( P \leq 0.005 \) was estimated by applying the method of Fernando et al. (2004). In addition to \( P \)-value analysis, 'fold-change' estimates and 95% confidence intervals associated with the fold-change estimates were computed as part of the mixed linear model analysis.

An additional 2754 spots were removed from the data set after data analysis because of concerns regarding the quality of the associated DNA sequences. Data (GEO GSE9430) reported in this study were derived from the remaining 7988 'informative' spots.

Semi-quantitative RT-PCR

Results from the microarray experiment were validated using an independent replicate of total RNA that was isolated from P6-stage primordia for L4 and L9. First-strand cDNA was synthesized using 1 \( \mu \)g of total RNA, 25 \( \mu \)g \( \cdot \) \( \mu \)l \(-1\) oligo(dT)22 primer, 0.5 mm deoxyribonucleotide triphosphate (dNTP) mix, 1x first-strand buffer, 0.01 \( \mu \)l DTT, 40 \( \mu \)l of RNaseOUT (Invitrogen) and 200 \( \mu \)l Super-Script II reverse transcriptase (Invitrogen) following the manufacturer’s directions. One-tenth of the reaction volume was used as the template for amplifications. The number of PCR cycles used for all genes is indicated in Figure 5. Primers used for amplifications are listed in Table S2.

RNA blot analysis

Total RNA (15 \( \mu \)g) per leaf was loaded in each lane of a 1% (w/v) agarose formaldehyde denaturing gel, and was blotted onto Hybond-N membranes (Amersham Biosciences, http://www.amershambiosciences.com). Filters were hybridized with DNA probes corresponding to the EST clamp on the array. EST-specific clones were generated by PCR filters were hybridized with DNA probes corresponding to ESTs. Amplified DNA products were purified using a QIAquick Gel Extraction kit (Qiagen, http://www.qiagen.com). DNA probes were labeled with 32P deoxyctydine triphosphate (dCTP) using a random prime method. The hybridization of probes and subsequent washings were performed as described by Church and Gilbert (1984), with slight modifications.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Expression sequence tags (ESTs) that were differentially expressed between juvenile leaf 4, adult leaf 9 and culture-rejuvenated leaves 3/4 in the high-intensity scan data set.

Table S1. Juvenile-induced expressed sequence tags (ESTs). These ESTs show at least a twofold increase in expression at \( P \leq 0.005 \) in juvenile tissues compared with adult tissues.

Table S2. Primers for the PCR amplification of selected phase-induced expressed sequence tags (ESTs).

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References


Vegetative phase change in maize


