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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 ≤0.005. 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 uronosyls 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 APETALA2 (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 targets 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).

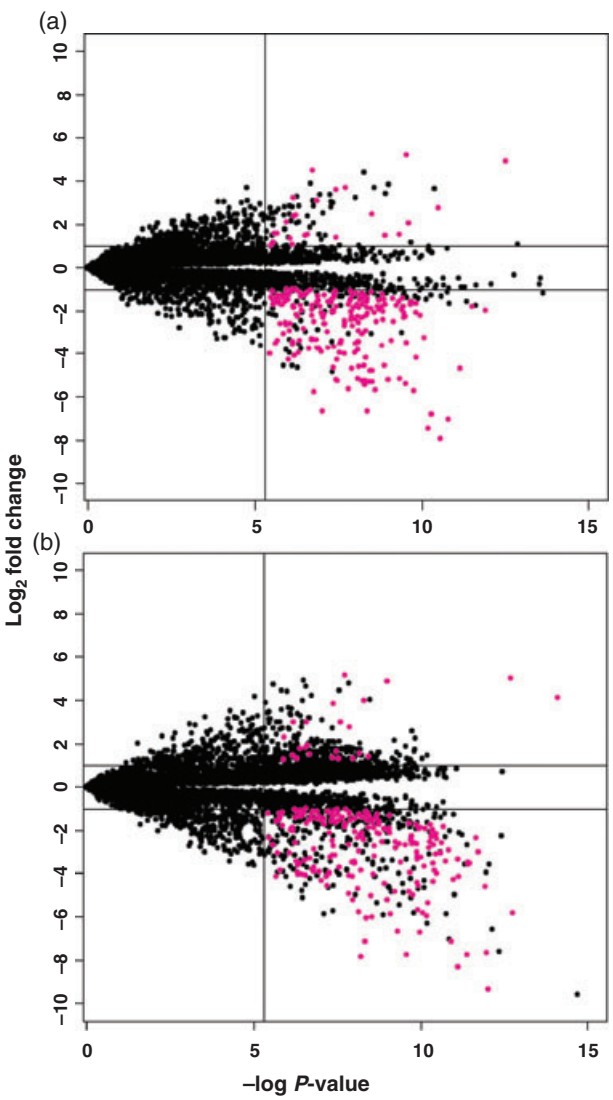

Figure 1 . Expressed sequence tags (ESTs) differentially expressed between juvenile leaf 4, adult leaf 9 and culture-rejuvenated leaves 3/4 in the low-intensity scan data set.
(a, b) Differences in EST expression levels were plotted against *P*-values derived from a Student's *t*-test. Horizontal lines indicate a difference in gene expression of twofold or more. The vertical line corresponds to a *P*-value of 0.005. Magenta dots in the lower and upper quadrants indicate juvenile-induced and adult-induced ESTs, respectively. The estimated false discovery rates were 2.2 and 0.5% for the data sets presented in (a) and (b), respectively.
(a) Adult leaf 9 compared with juvenile leaf 4.
(b) Adult leaf 9 compared with culture-rejuvenated leaves 3 or 4.

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

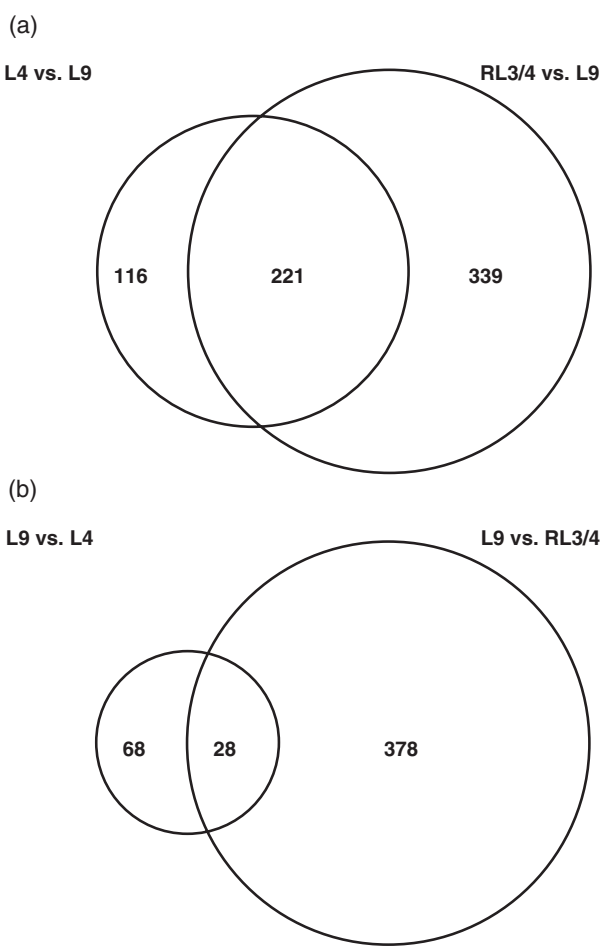

Figure 2. Venn diagrams of juvenile- and adult-induced data sets.
(a) Juvenile-induced expressed sequence tags (ESTs; overlap) derived from juvenile leaf 4 (L4) versus adult leaf 9 (L9) and culture-rejuvenated leaves 3 or 4 (RL3/4) versus L9 data sets.
(b) Adult-induced ESTs (overlap) derived from L9 versus L4 and L9 versus RL3/4 data sets.

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[a]

		Low-scan set[c]				High-scan set[c]			
		L4 versus L9[d]		RL3/4 versus L9[d]		L4 versus L9[d]		RL3/4 versus L9[d]	
EST GenBank AC	Top BLASTX annotation [GenBank AC (score; *e*-value; *species*)][b]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]
BM339253	Immunophilin, putative/FKBP-type pept idyl-prolyl cis-trans isomerase, putative [NP_568067.1 (172; 2e–41; *A. thaliana*)]	2.06E–05	132.00	1.83E–05	141.11	6.44E–04	10.46	4.65E–04	11.63
BM080125	Photosystem II subunit PsbS1 [NP_001105228.1 (73.9; 3e–12; *Z. mays*)]	2.36E–04	100.97	7.03E–05	214.72	8.03E–04	13.16	3.21E–04	18.48
BM074536	NADP-malic enzyme [AAP33011.1 (150; 7e–35; *Z. mays*)]	1.15E–03	55.49	2.41E–04	143.23	9.60E–04	6.39	4.68E–05	15.46
BG840776	One helix protein [AAM22751.1 (115; 2e–24; *D. antarctica*)]	2.52E–04	43.62	4.71E–05	104.48	6.79E–04	4.04	1.79E–05	9.00
BM267937	Photosystem-I reaction center subunit-II, chloroplast precursor (Photosystem-I 20-kDa subunit) (PSI-D) [P36213.1 (203; 4e–51; *H. vulgare*)]	5.67E–04	37.91	1.99E–04	64.80	3.77E–04	8.88	5.95E–05	15.73
DV550281	RISBZ4 [BAD26199.1 (157; 8e–37; *O. sativa*)]	5.94E–04	37.56	9.04E–05	101.79	–	–	–	–
BG840818	Putative protodermal factor [BAD26174.1 (93.2; 1e–17; *O. sativa*)]	1.26E–04	36.75	1.26E–04	36.76	7.40E–05	5.49	5.65E–05	5.81
DV491668	Chlorophyll *a*/*b* binding protein 48, chloroplast precursor (LHCII type-I CAB-48) (LHCP) [Q00827.1 (490; 1e–136; *Z. mays*)][g]	2.47E–04	18.15	5.86E–05	32.06	6.68E–04	2.76	2.90E–05	4.51
DV494632	Homeobox protein HD1, putative, expressed [ABF93721.1 (114; 2e–24; *O. sativa*)]	1.85E–03	15.49	1.63E–03	16.33	1.74E–03	2.47	8.25E–04	2.74
BM074151	ATNSI (NUCLEAR SHUTTLE INTERACTING); *N*-acetyltransferase [NP_973950.1 (88.2; 2e–16; *A. thaliana*)]	3.09E–04	14.03	1.88E–04	16.70	1.39E–03	2.84	1.47E–03	2.81
BM341648	Putative very-long-chain fatty acid condensing enzyme CUT1 [BAD15940.1 (175; 1e–42; *O. sativa*)]	9.98E–04	13.77	1.90E–03	10.89	4.57E–04	3.41	1.40E–03	2.85
BM334653	Ribulose bisphosphate carboxylase small subunit1 [NP_001105294.1 (268; 3e–69; *Z. mays*)][g]	5.29E–04	11.58	2.72E–04	14.52	2.00E–04	6.27	7.11E–05	8.07
BM074011	Chlorophyll *a*/*b* binding protein [AAC15992.1 (444; 5e–135; *O. sativa*)]	3.32E–04	11.38	5.11E–05	21.62	4.21E–04	8.04	4.40E–05	15.94
BM073471	Elongation factor family protein [NP_851035.1 (91.3; 5e–17; *A. thaliana*)]	3.02E–04	10.97	5.93E–04	8.92	5.73E–04	4.05	1.28E–03	3.49
BM073418	NDH-M (SUBUNIT NDH-M OF NAD(P)H:PLASTOQUINONE DEHYDRO-GENASE COMPLEX) [NP_001031804.1 (196; 7e–49; *A. thaliana*)]	1.09E–03	10.22	5.24E–04	13.11	8.49E–04	3.94	2.65E–04	4.98
BM350700	F-box family protein, putative, expressed [ABF93656 (101; 1e–19; *O. sativa*)][g]	–	–	–	–	8.90E–04	9.87	4.82E–03	5.87
BM075132	Pore-forming toxin-like protein Hfr-2 [AAW48295.1 (121; 9e–26; *T. aestivum*)][g]	1.25E–03	9.70	6.46E–05	28.47	1.61E–03	6.79	4.57E–05	21.22
BG842033	Catalase isozyme 3 [P18123.2 (201; 2e–50; *Z. mays*)]	2.87E–03	8.41	3.95E–04	16.97	4.44E–03	3.02	8.69E–05	6.75
BG840924	ABC transporter family protein [NP_175 837.2 (73.6; 6e–12; *A. thaliana*)]	7.73E–04	7.80	1.95E–03	6.02	–	–	–	–
DV489835	Putative photosystem-I reaction center subunit IV [BAC84088.1 (144; 2e–33; *O. sativa*)]	1.59E–03	7.10	3.61E–04	11.13	7.25E–04	2.61	2.15E–05	4.43
BG841780	Polyamine oxidase [CAC04002.1 (280; 6e–74; *Z. mays*)]	3.18E–03	6.06	6.89E–04	9.57	2.68E–03	5.68	5.26E–04	9.00

Table 1 *Continued*

EST GenBank AC	Top BLASTX annotation [GenBank AC (score; *e*-value; *species*)][b]	Low-scan set[c]				High-scan set[c]			
		L4 versus L9[d]		RL3/4 versus L9[d]		L4 versus L9[d]		RL3/4 versus L9[d]	
		P-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]
DV943320	Pyruvate, orthophosphate dikinase [AAA33498.1 (164; 2e–39; *Z. mays*)]	4.56E–04	5.73	3.18E–05	11.42	8.64E–04	5.01	4.95E–05	10.26
BM076073	Chalcone synthase C2 (Naringenin-chalcone synthase C2) [P24825.1 (85.1; 2e–15; *Z. mays*)]	–	–	–	–	2.95E–03	5.43	4.81E–03	4.78
DV491239	Serine/threonine kinase receptor precursor-like protein [BAC57307.1 (274; 4e–72; *O. sativa*)]	–	–	–	–	4.02E–03	5.14	4.61E–04	9.48
BM073392	Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II [AAK21880.1 (194; 4e–48; *H. vulgare*)]	1.88E–04	4.25	3.50E–05	5.92	1.87E–03	2.53	4.02E–04	3.17
DV492715	Pentatricopeptide (PPR) repeat-containing protein [NP_173324.1 (201; 1e–50; *A. thaliana*)]	9.20E–04	4.23	5.21E–05	8.09	–	–	–	–
DV491027	Putative ABC transporter [AAG52334.1 (80.1; 1e–13; *O. sativa*)]	6.60E–04	3.97	9.83E–05	5.84	–	–	–	–
DV491220	Restorer of fertility 2 [NP_001105891.1 (337; 4e–91; *Z. mays*)]	2.11E–03	3.55	7.05E–04	4.42	1.50E–03	2.10	8.93E–05	2.95
DV492988	Putative protein kinase [BAD94332.1 (193; 6e–48; *A. thaliana*)]	9.73E–04	3.49	5.63E–05	6.11	2.56E–03	2.56	9.06E–05	4.41
DV491620	Putative HECT ubiquitin-protein ligase 3 [BAD07806.1 (90.9; 2e–37; *O. sativa*)][g]	6.84E–04	3.06	4.43E–04	3.28	2.21E–03	2.24	2.55E–03	2.20
DV551035	Transposon protein, putative, CACTA, En/Spm sub-class, expressed [ABF97558.1 (254; 3e–66; *O. sativa*)]	3.51E–03	3.00	7.21E–05	6.49	3.51E–03	2.86	7.05E–05	6.00
DV492584	Triose phosphate/phosphate translocator, chloroplast precursor (cTPT) [P49133.1 (153; 1e–35; *Z. mays*)]	7.57E–04	2.98	1.20E–04	4.01	1.31E–03	2.87	1.16E–04	4.29
DV942293	Auxin response factor 2 [BAB85913.1 (189; 2e–46; *O. sativa*)]	2.91E–04	2.76	3.26E–04	2.72	2.58E–04	2.42	3.21E–04	2.36
BM351599	Ubiquitin family protein [NP_190104.1 (149; 7e–34; *A. thaliana*)][g]	2.83E–03	2.56	4.39E–04	3.43	–	–	–	–
DV490210	tpr domain containing protein [ABR25579.1 (323; 9e–87; *O. sativa*)]	8.50E–04	2.41	6.15E–04	2.51	–	–	–	–
AW042390	Steroid hormone receptor/transcription factor [NP_187714.1 (94; 4e–18; *A. thaliana*)]	–	–	–	–	1.84E–03	2.19	3.51E–03	2.04

[a]ESTs presented in this table are upregulated by at least twofold in both juvenile leaf 4 and rejuvenated leaves 3/4 compared with adult leaf 9 at $P \leq 0.005$.
[b]BLASTX screen of individual EST against the NCBI nr database (February 24, 2008); n.s., no significant BLAST hit (*e*-value cut-off of 1e–10).
[c]See Experimental procedures, Data acquisition.
[d]L4, leaf 4; RL3/4, rejuvenated leaves 3/4; L9, leaf 9.
[e]Individual *P*-values obtained from the normalized log-scale signal intensity with the mixed linear model.
[f]Antilog of estimated log scale expression differences.
[g]BLASTX screen of individual MAGIv4 (*e*-value cut-off of e–100) against the NCBI nr database (February 24, 2008).

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 seqeunce tags (ESTs) that are upregulated in adult leaf 9 compared with both juvenile leaf 4 and rejuvenated leaves 3/4[a]

		Low-scan set[c]				High-scan set[c]			
		L9 versus L4[d]		L9 versus RL3/4[d]		L9 versus L4[d]		L9 versus RL3/4[d]	
EST GenBank AC	Top BLASTX annotation [GenBank AC (score; *e*-value; *species*)][b]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]	*P*-value[e]	Ratio[f]
DV943135	Hypothetical protein OsJ_000126 [EAZ10301.1 (101; 6e–21; *O. sativa*)][g]	7.19E–05	36.80	1.26E–04	28.95	6.89E–04	2.89	1.46E–03	2.60
DV943306	60S ribosomal protein L27 (RPL27C) [NP_193236.1 (175; 3e–42; *A. thaliana*]	3.75E–06	30.39	3.13E–06	32.54	2.84E–03	2.02	9.76E–04	2.28
AI692021	Putative somatic embryogenesis protein kinase 1 [BAD68873.1 (119; 1e–25; *O. sativa*)]	1.20E–03	22.48	4.44E–04	35.67	3.17E–03	3.02	1.00E–03	3.72
DV493121	Os05g0356800 [NP_001055294.1 (136; 2e–29; *O. sativa*)][g]	4.52E–04	12.85	2.48E–04	15.86	–	–	–	–
AI734769	Class-III HD-Zip protein 4, putative, expressed [ABF97 828.1 (211; 1e–53; *O. sativa*)]	5.91E–04	11.99	2.09E–03	7.94	–	–	–	–
BM078628	Putative growth-regulating factor 13 [NP_001106044.1 (358; 4e–96; *Z. mays*)][g]	2.16E–03	9.25	6.20E–04	14.34	–	–	–	–
BM078110	WW domain containing protein, expressed [ABA96334.2 (135; 1e–30; *O. sativa*)]	1.06E–03	8.58	1.40E–03	7.88	–	–	–	–
DV489742	SEC14 cytosolic factor (secretion factor 14) family protein [CAJ75630.1 (155; 1e–36; *B. sylvaticum*)]	2.82E–05	6.79	7.61E–07	17.63	–	–	–	–
BM072783	n.s.	–	–	–	–	4.94E–03	5.89	3.19E–03	6.71
DY576313	Protein kinase family protein [NP_973956.1 (180; 1e–57; *A. thaliana*)][g]	2.04E–04	5.50	1.36E–03	3.73	–	–	–	–
DV493254	Protein kinase family protein [NP_566630.1 (135; 4e–29; *A. thaliana*)][g]	1.99E–03	5.45	5.00E–04	7.87	–	–	–	–
DV943127	Helix-loop-helix DNA-binding [ABD32380.2 (78.2; 3e–12; *M. truncatula*)][g]	2.12E–03	5.29	2.72E–03	4.97	1.25E–04	2.01	1.33E–04	2.00
DV943234	n.s.	2.62E–03	4.30	3.88E–04	6.79	5.83E–04	2.09	1.45E–04	2.43
AI737934	Putative lateral organ boundaries (LOB) domain family [AAT42184.1 (75.1; 5e–11; *Z. mays*)][g]	6.86E–05	4.22	2.76E–03	2.39	–	–	–	–
BM333901	n.s.	–	–	–	–	1.35E–03	3.44	3.82E–03	2.86
DV493108	n.s.	3.50E–03	2.99	1.45E–03	3.50	2.48E–03	2.91	1.39E–03	3.20
DV492422	Putative growth-regulating factor 13 [NP_001106044.1 (358; 4e–96; *Z. mays*)][g]	3.75E–03	2.97	1.72E–03	3.42	–	–	–	–
DV495600	Squamosa promoter-binding-like protein 13 [Q6Z461.1 (82; 9e–14; *O. sativa*)][g]	8.96E–05	2.94	3.47E–04	2.48	1.98E–04	2.53	5.83E–04	2.23
DV942770	n.s.	1.39E–03	2.86	2.12E–03	2.69	6.33E–04	2.57	1.15E–03	2.38
DV492592	Hypothetical protein OsJ_017000 [EAZ33517.1 (208; 2e–52; *O. sativa*)]	1.38E–04	2.83	2.20E–04	2.67	–	–	–	–
DV942169	Hypothetical protein Osl_016629 [EAY95396.1 (120; 7e–25; *O. sativa*)][g]	1.51E–03	2.79	1.29E–03	2.86	–	–	–	–
DV942475	Putative FH protein NFH2 [BAB86073.1 (189; 2e–46; *O. sativa*)]	5.93E–04	2.67	2.85E–04	2.96	4.85E–04	2.24	1.91E–04	2.48
BM075057	Cyclin-dependent kinases regulatory subunit [AAS13367.1 (144; 3e–33; *G. max*)]	2.35E–03	2.52	5.68E–04	3.12	–	–	–	–
BM073426	OSJNBa0041M21.1 [CAD40443.2 (199; 1e–49; *O. sativa*)]	–	–	–	–	3.01E–03	2.35	3.94E–03	2.27
DV942221	Hypothetical protein OsJ_002940 [EAZ13115.1 (85.9; 5e–27; *O. sativa*)][g]	–	–	–	–	1.82E–03	2.29	2.38E–04	2.99
BG842886	LIM transcription factor homolog [NP001104937.1 (239; 8e–71; *Z. mays*)]	3.88E–03	2.26	1.86E–03	2.50	2.83E–03	2.18	2.20E–03	2.25
DV489762	Unknown protein [NP_564481.1 (90.1; 7e–17; *A. thaliana*)]	2.22E–03	2.11	5.35E–04	2.49	–	–	–	–
BM072828	Inorganic pyrophosphatase [NP_001104889.1 (171; 1e–56; *Z. mays*)]	4.22E–03	2.05	6.46E–04	2.59	–	–	–	–

[a]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 $P \leq 0.005$.
[b]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).
[c]See Experimental procedures, Data acquisition.
[d]L9, leaf 9; L4, leaf 4; RL3/4, rejuvenated leaves 3/4.
[e]Individual *P*-values obtained from the normalized log-scale signal intensity with the mixed linear model.
[f]Antilog of estimated log scale expression differences.
[g]BLASTX screen of individual MAGIv4 (e-value cut-off e–100) against the NCBI nr (February 24, 2008).

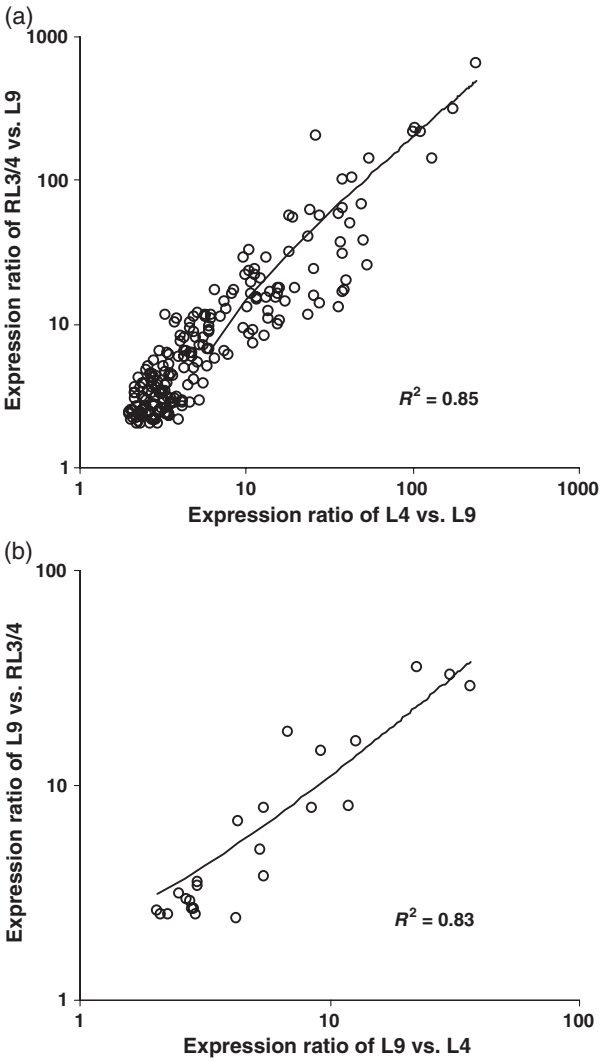

Figure 3. Expression ratios between juvenile leaf 4, adult leaf 9 and culture-rejuvenated leaves 3 or 4 comparisons in the low-intensity scan data set are highly correlated.
(a, b) Genes displaying the highest upregulation in each comparison are represented by the upper rightmost points. Note the log scale on both axes.
(a) Juvenile upregulated expressed sequence tags (ESTs). The mean ratio of the gene expression values was 0.91 ± 0.03 (n = 206; ±SEM).
(b) Adult upregulated ESTs. The mean ratio of the gene expression values was 0.97 ± 0.06 (n = 24).

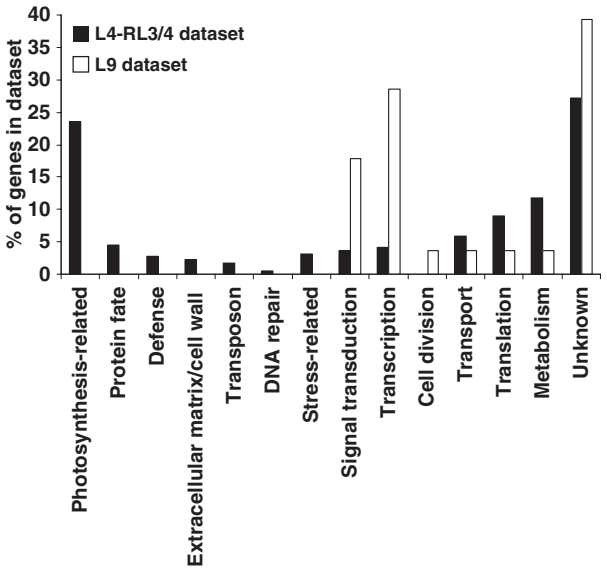

Figure 4. Functional categories of annotated expressed sequence tags (ESTs) in juvenile and adult upregulated data sets.
Bar heights indicate the number of ESTs assigned to each functional category as a percentage of the total number of genes in each data set. The ESTs were assigned to functional categories based on BLASTX annotation. The juvenile data set did not contain ESTs in the 'cell division' functional category, and the adult data set contained no ESTs in the 'photosynthesis-related', 'protein fate', 'defense', 'extracellular matrix/cell wall', 'transposon', 'DNA repair' or 'stress-related' functional categories.

sam.truman.edu/geneva/geneva.cgi; Buckner *et al.*, 2007) 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*

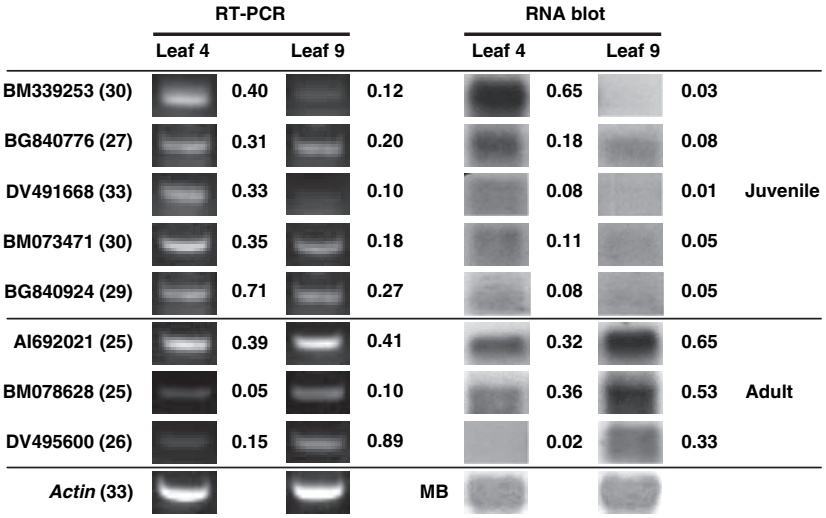

Figure 5. Corroboration of phase-induced differentially expressed genes.
Levels of mRNA isolated from leaf primordia were determined by semi-quantitative RT-PCR and RNA blot analyses. The GenBank accession number appears at the left-hand side. The number of PCR cycles is given in parentheses next to the GenBank accession number. The ratio of the signal relative to the loading control *Actin* or methylene blue (MB) staining of the 28S rRNA for leaf 4 or leaf 9 is shown to the right-hand side of each RT-PCR or RNA blot image, respectively. Juvenile expressed sequence tags (ESTs) that were upregulated in both leaf 4 (L4) and in culture-rejuvenated leaves 3 or 4 (RL3/4) compared with adult leaf 9 (L9); adult ESTs that were upregulated in L9 compared with both L4 and RL3/4.

(*ZmFkb*; BM339253) and putative *chlorophyll a/b binding protein 48* (*Cab48*; DV491668), and the adult upregulated putative s*omatic embryogenesis protein kinase 1* (*ZmSek*; AI692021) and putative *squamosa promoter binding-like protein 13* (*ZmSpl*; DV495600).

Expression analysis of phase-induced genes in successive leaf primordia from wild-type maize plants

Leaves 4 and 9 were selected as representatives of the juvenile and the adult phases, respectively, for microarray analysis. Whether gene expression in juvenile or adult leaves at other nodes within the two phases of a seed-derived plant is accurately represented by the expression profiles generated for leaves 4 or 9 could not be assessed by this profiling strategy. To obtain a more accurate picture of the expression of *ZmFkb*, *Cab48*, *ZmSek* and *ZmSpl* in the juvenile and adult phases, RNA blot assays were performed on P6-stage primordia of juvenile (1–4), transition (5–7) and adult (8–14) leaves from wild-type plants. In wild-type maize plants, transcripts for the juvenile upregulated *ZmFkb* were most abundant in juvenile leaf 1, and decreased precipitously in successive juvenile leaves (Figure 6a,c). *ZmFkb* expression was not detected in transition (5–7) or adult (8–14) leaves. Expression of the juvenile upregulated *Cab48* was, like *ZmFkb*, strongest in leaf 1, and steadily diminished in subsequent leaves, so that by mid-transition (leaf 6), mRNA levels were uniformly at the threshold of detection by RNA blot analysis (Figure 6b,d). Transcript levels of the adult upregulated *ZmSek* were moderately low in juvenile leaves 1–4 and transition leaves 5–7 (Figure 6a,e), but started to increase at leaf 8, the first adult leaf, accumulated to their highest level in leaf 10 and declined in later adult leaves. The adult upregulated *ZmSpl* mRNA was virtually undetectable in juvenile leaves 1–4. *ZmSpl* transcript levels increased beginning with transition leaf 5, and accumulated to high levels in adult leaves 10–14 (Figure 6b,f); in leaves 10 and 11, a second, higher molecular weight transcript was apparent. Thus, whereas some of the microarray identified genes (*ZmSek* and *Cab48*) simply showed higher expression in leaf primordia of one phase compared with the other, 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,

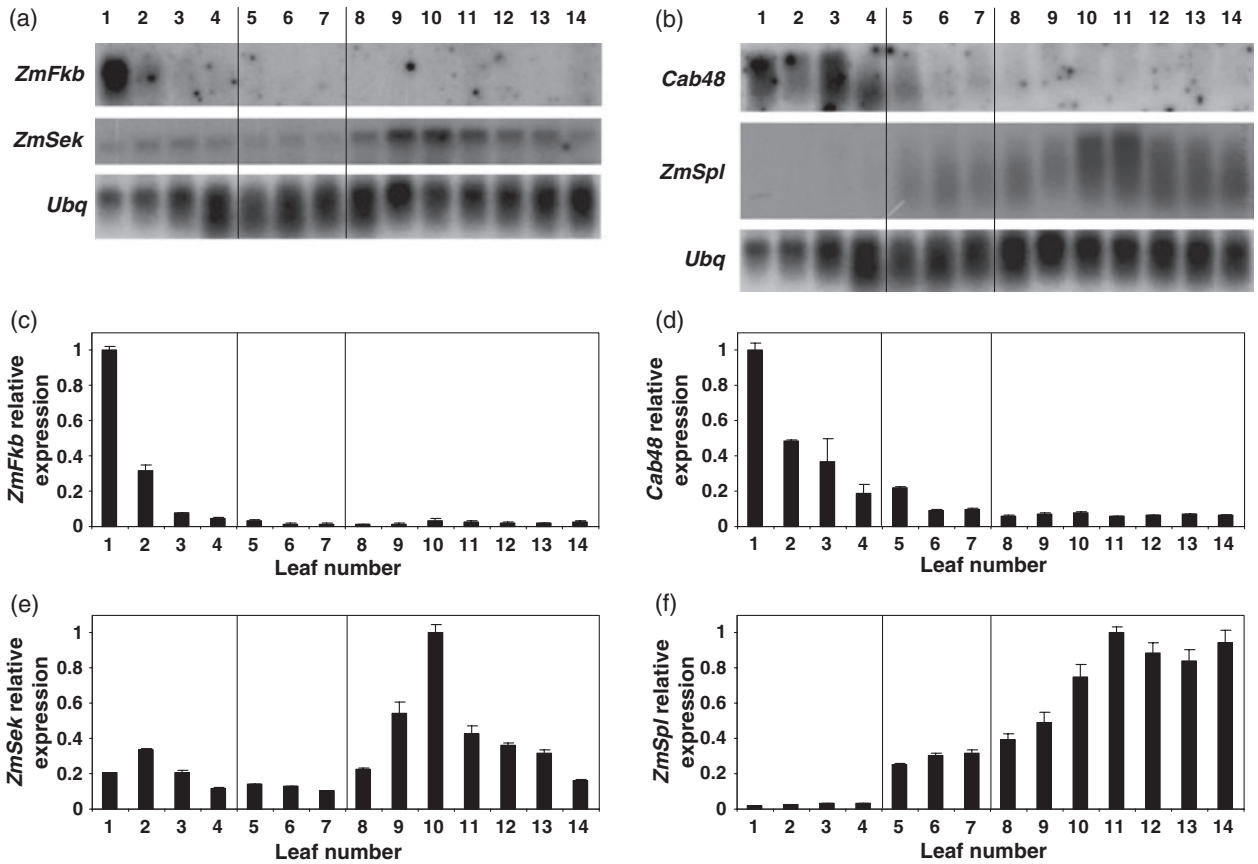

Figure 6. *ZmFkb*, *Cab48*, *ZmSek* and *ZmSpl* expression in successive leaf primordia of wild-type plants. RNA was isolated from plastochron 6 (P6)-staged primordia from wild-type plants.

The leaf number is indicated above each lane. Vertical lines separate juvenile, transition and adult samples. Total RNA (15 μg) was loaded per sample. The intensities of the bands in (c–f) were quantified by using NIH ImageJ (http://rsbweb.nih.gov/ij), and were normalized to *Ubiquitin*. The expression levels were quoted relative to the most intense band among individual leaves, arbitrarily set to a value of 1.0. The error bars indicate the standard errors of the mean (n = 2). (a, c) *ZmFkb*; (a, e) *ZmSek*; (b, d) *Cab48*; (b, f) *ZmSpl*.

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; Orkwiszewski 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 FKBP and cylophilin families function similarly to chaperones in assisting protein folding (Romano *et al.*, 2005). FKBP and cyclophilin proteins have an N-terminal peptidyl prolyl isomerase (PPIase) domain and three tetratricopeptide (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 $Cg1^+$ encodes an *miR156* RNA (Chuck *et al.*, 2007). Thirteen potential *SPL* genes, including *tga*, that are expected to be targets of $Cg1^+$ 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 F_1 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 undefined. This 5/8 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 L8 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 (Wolfinger *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 7886 '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 μg of total RNA, 25 μg ml^{-1} oligo$(dT)_{22}$ primer, 0.5 mM deoxyribonucleotide triphosphate (dNTP) mix, 1× first-strand buffer, 0.01 M DTT, 40 U of RNaseOUT (Invitrogen) and 200 U of Superscript II reverse transcriptase (Invitrogen) following the manufacturer's directions. One-twentieth 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 μg) 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.amersham.com). Filters were hybridized with DNA probes corresponding to the EST clone on the array. EST-specific clones were generated by PCR amplification of the cDNA insert using gene-specific primers (IDT, http://eu.idtdna.com; Table S2) in reactions with 1× PCR buffer, 0.2 μM of each primer, 0.2 mM dNTPs and 0.04 U of Taq polymerase (NEB, http://www.neb.com). Thirty-five cycles were used to amplify all ESTs. Amplified DNA products were purified using a QIAquick Gel Extraction kit (Qiagen, http://www.qiagen.com). DNA probes were labeled with [α-^{32}P] deoxycytidine 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.

Acknowledgements

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Supporting Information

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

Figure S1. Expressed 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

Abedon, B.G., Hatfield, R.D. and Tracy, W.F. (2006) Cell wall composition in juvenile and adult leaves of maize (*Zea mays* L.). *J. Agric. Food. Chem.* **54**, 3896–3900.

Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. *Plant Cell*, **15**, 2730–2741.

Berardini, T.Z., Bollman, K., Sun, H. and Poethig, R.S. (2001) Regulation of vegetative phase change in *Arabidopsis thaliana* by cyclophilin 40. *Science*, **291**, 2405–2407.

Bongard-Pierce, D.K., Evans, M.M.S. and Poethig, R.S. (1996) Heteroblastic features of leaf anatomy in maize and their genetic regulation. *Int. J. Plant Sci.* **157**, 331–340.

Brink, R.A. (1962) Phase change in higher plants and somatic cell heredity. *Q. Rev. Biol.* **37**, 1–22.

Brusslan, J.A. and Tobin, E.M. (1992) Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings. *Proc. Natl Acad. Sci. USA*, **89**, 7791–7795.

Buckner, B., Beck, J., Browning, K. *et al.* (2007) Involving undergraduates in the annotation and analysis of global gene expression studies: creation of a maize shoot apical meristem expression database. *Genetics*, **176**, 741–747.

Chang, Y.C. and Walling, L.L. (1992) Chlorophyll *a*/*b*-binding protein genes are differentially expressed during soybean development. *Plant Mol. Biol.* **19**, 217–230.

Chory, J., Nagpal, P. and Peto, C.A. (1991) Phenotypic and genetic analysis of *det2*, a new mutant that affects light-regulated seedling development in *Arabidopsis*. *Plant Cell*, **3**, 445–459.

Christensen, A.H. and Quail, P.H. (1989) Sequence analysis and transcript regulation by heat shock of polyubiquitin transcripts from maize. *Plant Mol. Biol.* **12**, 619–632.

Chuck, G., Cigan, A.M., Saeteurn, K. and Hake, S. (2007) The heterochronic maize mutant *Corngrass1* results from overexpression of a tandem microRNA. *Nat. Genet.* **39**, 544–549.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Dudoit, S., Yang, Y.H., Callow, M.J. and Speed, T.P. (2002) Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. *Stat. Sin.* **12**, 111–139.

Evans, M.M.S. and Poethig, R.S. (1995) Gibberellins promote vegetative phase change and reproductive maturity in maize. *Plant Physiol.* **108**, 475–487.

Evans, M.M.S. and Poethig, R.S. (1997) The *viviparous8* mutation delays vegetative phase change and accelerates the rate of seedling growth in maize. *Plant J.* **12**, 769–779.

Evans, M.M.S., Passas, H. and Poethig, R.S. (1994) Heterochronic effects of *glossy15* mutations on epidermal cell identity in maize. *Development*, **120**, 1971–1981.

Fernando, R.L., Nettleton, D., Southey, B.R., Dekkers, J.C.M., Rothschild, M.F. and Soller, M. (2004) Controlling the proportion of false positives in multiple dependent tests. *Genetics*, **166**, 611–619.

Freeling, M. (1992) A conceptual framework for maize leaf development. *Dev. Biol.* **153**, 44–58.

Freeling, M. and Lane, B. (1994) The maize leaf. In *The Maize Handbook* (Freeling, M. and Walbot, V., eds). New York: Springer Verlag, pp. 17–28.

Gandikota, M., Birkenbihl, R.P., Hohmann, S., Cardon, G.H., Saedler, H. and Huijser, P. (2007) The miRNA 156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. *Plant J.* **49**, 683–693.

Hackett, W.P. (1985) Juvenility, maturation and rejuvenation in woody plants. *Hortic. Rev.* **7**, 109–155.

He, Z.H., Li, J., Sundqvist, C. and Timko, M.P. (1994) Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). *Plant Physiol.* **106**, 537–546.

Irish, E.E. and Karlen, S. (1998) Restoration of juvenility in maize shoots by meristem culture. *Int. J. Plant Sci.* **159**, 695–701.

Irish, E.E. and McMurray, D. (2006) Rejuvenation by shoot apex culture recapitulates the developmental increase of methylation at the maize gene *Pl-Blotched*. *Plant Mol. Biol.* **60**, 747–758.

Irish, E.E. and Nelson, T. (1988) Development of maize plants from cultured shoot apices. *Planta*, **175**, 9–12.

Kerstetter, R.A. and Poethig, R.S. (1998) The specification of leaf identity during shoot development. *Annu. Rev. Cell Dev. Biol.* **14**, 373–398.

Langdale, J.A., Rothermel, B.A. and Nelson, T. (1988) Cellular patterns of photosynthetic gene expression in developing maize leaves. *Genes Dev.* **2**, 106–115.

Lauter, N., Kampani, A., Carlson, S., Goebel, M. and Moose, S.P. (2005) *microRNA172* down-regulates *glossy15* to promote vegetative phase change in maize. *Proc. Natl Acad. Sci. USA*, **102**, 9412–9417.

Millar, A.A., Clemens, S., Zachgo, S., Giblin, E.M., Taylor, D.C. and Kunst, L. (1999) *CUT1*, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *Plant Cell*, **11**, 825–838.

Moose, S.P. and Sisco, P.H. (1994) *Glossy15* controls the epidermal juvenile-to-adult phase transition in maize. *Plant Cell*, **6**, 1343–1355.

Moose, S.P. and Sisco, P.H. (1996) *Glossy15*, an *APETALA2*-like gene from maize that regulates leaf epidermal cell identity. *Genes Dev.* **10**, 3018–3027.

Moreno, M.A., Harper, L.C., Krueger, R.W., Dellaporta, S.L. and Freeling, M. (1997) *liguleless1* encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. *Genes Dev.* **11**, 616–628.

Nakazono, M., Qiu, F., Borsuk, L.A. and Schnable, P.S. (2003) Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. *Plant Cell*, **15**, 583–596.

Orkwiszewski, J. and Poethig, R.S. (2000) Phase identity of the maize leaf is determined after leaf initiation. *Proc. Natl Acad. Sci. USA*, **97**, 10631–10636.

Pighin, J.A., Zheng, H., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L. and Samuels, A.L. (2004) Plant cuticular lipid export requires an ABC transporter. *Science*, **306**, 702–704.

Poethig, R.S. (1988) Heterochronic mutations affecting shoot development in maize. *Genetics*, **119**, 959–973.

Poethig, R.S. (1990) Phase change and the regulation of shoot morphogenesis in plants. *Science*, **250**, 923–930.

Pratt, W.B., Krishna, P. and Olsen, L.J. (2001) Hsp90-binding immunophilins in plants: protein movers. *Trends Plant Sci.* **6**, 54–58.

Ramsperger, V.C., Summers, R.G. and Berry, J.O. (1996) Photosynthetic gene expression in meristems and during initial leaf development in a C_4 dicotyledonous plant. *Plant Physiol.* **111**, 999–1010.

Reimer, U. and Fischer, G. (2002) Local structural changes caused by peptidyl prolyl *cis*/*trans* isomerization in the native state of proteins. *Biophys. Chem.* **96**, 203–212.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P. (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513–520.

Romano, P., Gray, J., Horton, P. and Luan, S. (2005) Plant immunophilins: functional versatility beyond protein maturation. *New Phytol.* **166**, 753–769.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M. and Weigel, D. (2005) Specific effects of microRNAs on the plant transcriptome. *Dev. Cell*, **8**, 517–527.

Simpson, G.G., Gendall, A.R. and Dean, C. (1999) When to switch to flowering. *Annu. Rev. Cell Dev. Biol.* **15**, 519–550.

Skibbe, D.S., Wang, X., Zhao, X., Borsuk, L.A., Nettleton, D. and Schnable, P.S. (2006) Scanning microarrays at multiple intensities enhances discovery of differentially expressed genes. *Bioinformatics*, **22**, 1863–1870.

Spencer, M.W.B., Casson, S.A. and Lindsey, K. (2007) Transcriptional profiling of the Arabidopsis embryo. *Plant Physiol.* **143**, 924–940.

Steeves, T.A. and Sussex, I.M. (1988) *Patterns of Plant Development*, 2nd edn. New York: Cambridge University Press.

Vega, S.H., Sauer, M., Orkwiszewski, J.A.J. and Poethig, R.S. (2002) The *early phase change* gene in maize. *Plant Cell*, **14**, 133–147.

Wang, H., Nussbaum-Wagler, T., Li, B., Zhao, Q., Vigouroux, Y., Faller, M., Bomblies, K., Lukens, L. and Doebley, J.F. (2005) The origin of the naked grains of maize. *Nature*, **436**, 714–719.

Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeth, L., Bushel, P., Afshari, C. and Paules, R.S. (2001) Assessing gene significance from cDNA microarray expression data via mixed models. *J. Comput. Biol.* **8**, 625–637.

Woo, H.-H., Hackett, W.P. and Das, A. (1994) Differential expression of a chlorophyll a/b binding protein gene and a proline rich gene in juvenile and mature phase English ivy (*Hedera helix*). *Physiol. Plant*, **92**, 69–78.

Wu, G. and Poethig, R.S. (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by *miR165* and its target *SPL3*. *Development*, **133**, 3539–3547.

Xie, K., Wu, C. and Xiong, L. (2006) Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant Physiol.* **142**, 280–293.

