

Expression Profiling of Reciprocal Maize Hybrids Divergent for Cold Germination and Desiccation Tolerance

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Recombinant inbred lines (RILs) derived from B73 × M017 were screened for cold germination (CG) and desiccation tolerance (DT) phenotypes. Reciprocal F₁ hybrids were made between divergent RILs, and hybrids that showed differential phenotypes (parent-of-origin effect) for CG or DT were selected for profiling mRNA and protein expression. mRNA and proteins were extracted from embryo axes of seed germinated for 11 d at 12.5°C in the dark and developing embryos at 40% seed moisture (R5 stage) for CG and DT, respectively. GeneCalling analysis, an open-ended mRNA profiling method, identified 336 of 32,496 and 656 of 32,940 cDNA fragments that showed ≥1.5-fold change in expression between the reciprocal F₁ hybrids for CG and DT, respectively. Protein expression map (PEM) analysis, an open-ended two-dimensional polyacrylamide gel electrophoresis, identified 117 of 2,641 and 205 of 1,876 detected proteins to be differentially expressed with ≥1.5-fold change between the reciprocal F₁ hybrids in CG and DT samples, respectively. A subset of these proteins was identified by tandem mass spectrometry followed by database query of the spectra. The differentially expressed genes/proteins were classified into various functional groups including carbohydrate and amino acid metabolism, ion transporters, stress and defense response, polyamine metabolism, chaperonins, cytoskeleton associated, etc. Phenotypic analysis of seed from self-pollinated ears of the reciprocal F₁ hybrids displayed small differences compared with the reciprocal hybrids themselves, suggesting a negligible effect of cytoplasmic factors on CG and DT traits. The results provide leads to improving our understanding of the genes involved in stress response during seed maturation and germination.

There have been several reports where differential phenotypic expression was observed between reciprocal F₁ hybrids in maize (*Zea mays*) for various kernel and germination traits such as whole-kernel growth rate (Groszmann and Sprague, 1948), embryo and endosperm dry weight (Bagnara and Daynard, 1983), embryo oil and protein (Miller and Brimhall, 1951), zein synthesis (Chaudhuri and Messing, 1994), seed germination at low temperatures (Pinnell, 1949), and tolerance to drying injury (Bdliya and Burris, 1988). These differential expressions can be attributed to epigenetic phenomena such as genomic imprinting (described below) and xenia (refers to the effect of pollen on the endosperm phenotype in the same generation), dosage effects (in case of triploid tissue such as endosperm), and cytoplasmic effects (so-called maternal effects from mitochondrial and chloroplast genomes or nuclear-encoded cytoplasmically stored gene products).

Genomic imprinting, also referred to as uniparental dominance or parent-of-origin effect, is a well-studied phenomenon in both mammals and plants (for reviews, see Alleman and Doctor, 2000; Reik and Walter, 2001). Imprinting refers to a reversible epigenetic modification of loci resulting in differential expression of genes depending on the parent of origin.

Unlike Mendelian inheritance, a given allele of an imprinted gene is phenotypically expressed in the F₁ depending on whether it is transmitted through the male or female parent. Imprinting in mammals occurs primarily via differential DNA methylation and alteration in chromatin structure resulting in silencing of specific allele (see Feil and Khosla, 1999; Tilghman, 1999). Possible roles of differential methylation in imprinting of zein and α -tubulin genes in maize (Lund et al., 1995a, 1995b) and chromatin remodeling (structural alteration in chromatin) in regulating imprinting of MEDEA (*MEA*) locus in Arabidopsis (Jeddeloh et al., 1999; Vielle-Calzada et al., 1999) have been reported. However, the specific role of DNA methylation-mediated gene silencing in imprinting of genes during embryo and endosperm development in plants is not clear (Vielle-Calzada et al., 1999; Luo et al., 2000; Russinova and de Vries, 2000).

Comparison of reciprocal F₁ hybrids provides an excellent system to study genes associated with parent-of-origin effects (some of which may be due to genomic imprinting) in diploid tissue that influences various traits of agronomic interest. In hybrid seed production systems such as maize, sorghum, and millets, identification of genes associated with imprinting provides an opportunity to select parents (male or female) through which to introduce the desired alleles.

Northern corn-growing regions have a short crop-growing season. This often requires planting of seed

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early in the season, when soil temperatures are low, followed by early season harvesting to avoid frost injury. Therefore, it is important for grain to be used as seed to tolerate artificial drying (desiccation) when harvested at high seed moisture ($\geq 40\%$) and to retain the ability to germinate under cold conditions for better field stand establishment. Extensive efforts have been devoted to understanding the genetics and physiology of the cold germination (CG) and desiccation tolerance (DT) traits (Burris, 2000; Hoegemeyer and Gutormsen, 2000). Changes in various compositional (oligosaccharides, lipids, hormones, enzymes, free radicals, and metabolic intermediates) and organellar/suborganellar properties (mitochondria, oil and protein bodies, starch grains, membrane integrity, and structure of chromatin and proteins) in the seed and germinating seedlings of maize have been reported to be associated with CG (Yacoub and Fillion, 1986; Janowiak and Markowski, 1987; Stewart et al., 1990a, 1990b; Schell et al., 1991; Prasad, 1996, 1997; Santis et al., 1999) and DT (Leprince et al., 1993, 1994, 1995; Bochicchio et al., 1994a, 1994b; Oishi and Bewley, 1990; Chen and Burris, 1991; Brenac et al., 1997; Obendorf, 1997; Perdomo and Burris, 1998; Wolkers et al., 1998).

Much of the literature relevant to cold stress response at the molecular level comes from *Arabidopsis* where several cold-regulated (COR) genes induced in response to abscisic acid (ABA) were characterized (for review, see Thomashow, 1999). An ABA-independent low-temperature responsive regulatory cascade involving a sequential expression of C-repeat (CRT)/dehydration responsive element (DRE) binding factors genes, followed by CRT/DRE-containing COR genes was recently characterized in *Arabidopsis* (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000). Overexpression of CRT/DRE binding factor-1 or DREB1A under the control of cauliflower mosaic virus 35S or stress inducible rd29A promoters was found to induce COR genes enhancing freezing tolerance in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Seki et al. (2001) reported a genome-wide survey of mRNA expression pattern of approximately 1,300 genes, using cDNA microarray, in *Arabidopsis* subjected to drought and cold stress at various stages from germination to maturity. They identified 44 and 19 cDNAs that were inducible by drought and cold stress, respectively, of which 12 were DREB1A target genes. Such detailed molecular studies of cold and desiccation stress responses have not been reported in maize.

The objective of this study was to identify differentially expressed genes related to CG and DT phenotypes. We have identified phenotypically divergent recombinant inbred lines (RILs) from the cross B73 \times M017 (SX-19) for CG and DT and produced reciprocal F_1 hybrids. The reciprocal F_1 hybrids that showed differential phenotypes for CG and DT, an

indication of potential imprinting effects (or nuclear cytoplasmic effects), were profiled for mRNA and protein expression using open-ended platforms. This approach allowed the profiling of genetically identical material thus enhancing the effectiveness of expression profiling.

RESULTS

Screening, Crossing, and Phenotypic Analysis

Five RILs that showed high germination ($>90\%$) and five RILs that showed low germination ($\leq 30\%$) under cold test conditions were identified. Similarly, five each of tolerant and sensitive RILs to high-temperature desiccation were identified. All genotypes germinated normally at ambient temperatures (data not shown). Reciprocal F_1 hybrids produced in a five-by-five diallel-crossing scheme, showed varying degrees of responses within each trait (data not shown). The F_1 hybrids displayed transgressive (heterosis) and additive responses compared with their self-pollinated parental phenotypes. In instances where the reciprocal F_1 hybrids showed differential phenotypes, the phenotype of the F_1 s with the high parent (i.e. parent with high germination score) as the maternal parent was typically higher in their germination scores compared with those with low parents as the maternal parent. Maternal dominance was clearly evident among some, but not all, RIL combinations for both traits. Of all of the possible RIL combinations tested within each trait, at least six and five showed parent-of-origin effect for CG and GT, respectively (data not shown). Because of the complexity and resource requirements of the profiling methods used, only one representative RIL combination from each trait was selected for expression profiling (Figs. 1–3). We commonly observed among the data, as demonstrated for CG in Figure 1, that the phenotype of the F_1 hybrid (i.e. M0023-syn0 \times M0081-syn0), which involved the low-phenotype parent (i.e. M0023-syn0) as a female, was typically close to the mid-parent value. This pattern of maternal influence attributable to parent-of-origin effect was previously observed in other studies involving

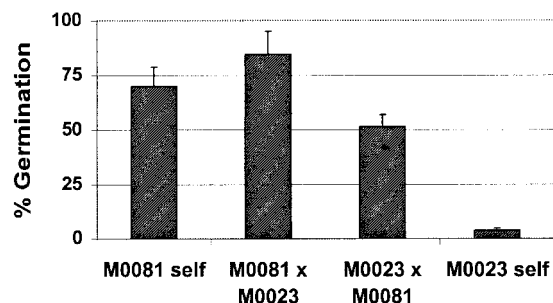


Figure 1. CG response of self-pollinated RILs and their reciprocal F_1 hybrids. The differential response of the reciprocal F_1 hybrids indicates maternal dominance (parent-of-origin effect).



Figure 2. Seeds of reciprocal F₁ hybrids germinated for 11 d at 12.5°C in the dark. Top panel, F₁ (M0081-syn0 × M0023-syn0); bottom panel, F₁ (M0023-syn0 × M0081-syn0). Parents, M0081-syn0, and M0023-syn0 showed a similar response to that of F₁-M0081-syn0 × M0023-syn0 and the F₁-M0023-syn0 × M0081-syn0, respectively (data not shown).

maize kernel growth and its components (Groszmann and Sprague, 1948; Bagnara and Daynard, 1983). Phenotypic differences between the reciprocal F₁ hybrids could also be clearly seen when the seeds were germinated at 12.5°C for 11 d in the dark (Fig. 2).

When the seeds were harvested at high moisture (40%) and dried at high temperature (43°C), the phenotypic differences between the reciprocal F₁s in the DT experiment was similar to those in the CG experiment, with one exception. The phenotype of the F₁ M0337-syn4 × M0021-syn4 having the low-DT parent (M0337-syn4) as female did not approach the mid-parent value (Fig. 3). Although the reciprocal F₁ hybrids were significantly different in their phenotype after drying at high (43°C) temperature, the phenotypic differences were not significant after drying at the low (35°C) temperature (Fig. 3). When the reciprocal F₁ hybrids of CG and DT traits were self-pollinated in the subsequent year, the magnitude of phenotypic differences between them was

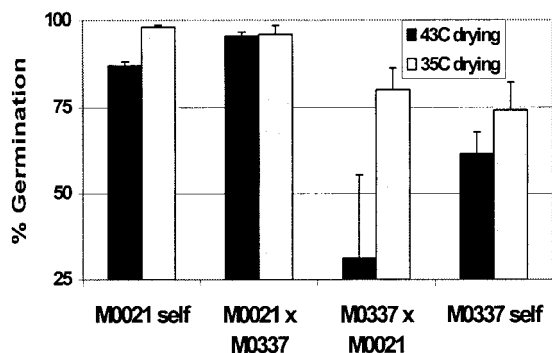


Figure 3. CG response of self-pollinated RILs and their reciprocal F₁s after high-moisture harvest and high- or low-temperature drying. The differential response of the reciprocal F₁ hybrids at high-temperature (43°C) drying but not at low-temperature (35°C) drying indicates maternal dominance (parent-of-origin effect).

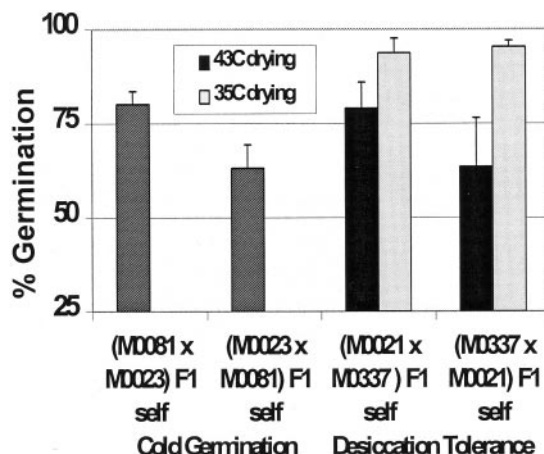


Figure 4. CG response of self-pollinated reciprocal F₁ hybrids. The phenotypic differences between the self-pollinated reciprocal F₁ hybrids were significantly reduced for both CG and DT traits. This suggests that the phenotypic differences between the reciprocal F₁ hybrids were mainly due to nuclear genes rather than cytoplasmic (mitochondrial or chloroplast genomes) factors.

noticeably reduced (Fig. 4). This suggests that the nuclear genome played a major role in determining phenotypic differences observed in the F₁ generation, rather than cytoplasmic (mitochondrial and chloroplast) genes.

Transcript Profiling

cDNAs synthesized from poly(A⁺) RNA of germinating embryo axes (CG) and embryos at 40% moisture content (DT) were digested with unique pairs of restriction enzymes in separate reactions. cDNA restriction fragments resolved by capillary gel electrophoresis varied in length from 40 to 450 nt. Use of 89 and 88 restriction enzyme pairs yielded a total of 32,496 and 32,940 detectable cDNA fragments in CG and DT samples, respectively. Quantitative expression analysis of the individual fragments, as shown by the peak heights in the electrophoretic scans, revealed that only 336 (1%) and 656 (2%) of the cDNA fragments were differentially expressed with ≥1.5-fold change between the reciprocal F₁ hybrids in CG and DT traits, respectively.

GeneCalling analysis identified the genes that matched the cDNA restriction fragments against the predicted fragments in the public and proprietary databases. Most of the fragments matched more than one gene in the databases. Also, many genes were “called” by more than one cDNA restriction fragment. A subset of the cDNA fragments and their corresponding gene matches were selected for confirmation through cloning, sequencing, and competitive PCR analyses. The selection was based on whether the fragment showed a high degree of fold change between the reciprocal F₁ hybrids and/or whether multiple cDNA fragments, with a consistent pattern of differential expression (measured by *t*

test), were indicated for a given gene. In cases where the same gene matched multiple cDNA fragments with similar levels of expression, only one of the fragments was included for analysis. The confirmed genes with their function, fold change in expression, and cDNA restriction fragment size are listed in Ta-

ble I for CG and in Table II for DT. The gene names and accession numbers were obtained using BLAST search of the public databases (GenBank and SwissProt/TrEMBL) with probability value of $\leq 1 \times 10^{-07}$. Competitive PCR analysis using sequence-specific primers was used for sequence confirmation

Table 1. Differentially expressed genes for the CG trait revealed by mRNA profiling

The expression levels of mRNA extracted from embryo axes tissue of seeds germinated for 11 d at 12.5°C in the dark were compared between the reciprocal F₁ hybrids that were high (M0081-syn0 × M0023-syn0) and low (M0023-syn0 × M0081-syn0) for CG phenotype. The gene/maize EST identities of the cDNA fragments were obtained by BLAST search of the GenBank and SwisProt/TrEMBL databases with a probability value of $\leq 1.0 \times 10^{-7}$.

Change ^a	Accession No. ^b	Percent Identity ^c	cDNA Size ^d	Function ^e	Gene Description or Source of EST ^f
<i>-fold</i>			<i>nt</i>		
1.5	Q42972; A1637077	78; 98	286	Carbohydrate metabolism	Malate dehydrogenase, glyoxysomal (EC 1.1.1.37); Stressed shoot cDNA library
5	U27116	94	278	Cell wall structure	Caffeoyl-CoA 3-O-methyltransferase
1.7	O24496; AA979807	79; 98	138	Cellular detoxification	Glyoxalase II (cytoplasmic isozyme, EC 3.1.2.6); green seedling cDNA library
-3.3	AF077407	100	113	Chaperonin	Chaperonin containing TCP-1 complex γ -chain
2.1	Q9SL04; AW146747	85; 97	446	Cytoskeletal structural protein	Microtubule-associated protein; root cDNA library
-1.8	X15704	100	427	Cytoskeletal structural protein	Tubulin (α 1 and 2)
2.8	X57313	100	75	DNA synthesis and replication	H2B histone
1.6	Q9LT02	80	354	Ion channel	Cation-transporting ATPase (EC 3.6.3.-)
2.7	AF089838	100	80	Lipid and sterol metabolism	Phosphatidylglycerol/phosphatidylinositol transfer protein
14.5	AF085149	100	74	Protein destination and storage	Aminotransferase
1.9	AF033496	100	314	Secondary metabolism (phenolic O-methyl transferase)	Herbicide safener binding protein (SBP1)
5	Y07767	100	59	Secondary metabolism (polyamine)	S-Adenosylmethionine decarboxylase
2.3	AF039573	100	165	Stress response	ABA- and stress-inducible protein (Asr1)
1.9	Q9XFH5	73	122	Sulfur metabolism	Alliinase (EC 4.4.1.4)
-1.5	AF091837	77	328	Transcription factor	DNA-binding protein
3.7	X01365	98	79	Unclassified	Chloroplast DNA for 4.5S and 23S ribosomal RNA
2.1	Z97336	98	175	Unclassified	Hypothetical protein (Arabidopsis)
1.7	AL035656	98	156	Unclassified	Putative protein (Arabidopsis)
1.6	AC004218	98	197	Unclassified	Medicago nodulin N21-like protein (Arabidopsis)
1.5	BE638609	100	349	Unclassified	PRT1 protein (tobacco [<i>Nicotiana tabacum</i>])
-1.6	A1622713	100	94	Unclassified	Leaf primordia cDNA library
-1.5	BAB08430	81	336	Unclassified	GAMM1 protein-like
-1.7	D48425	84	292	Unclassified	Similar to rice (<i>Oryza sativa</i>) RICS14613A rice green shoot <i>O. sativa</i>

^a The values, positive and negative, represent fold changes of mRNA abundance in high versus low reciprocal F₁ hybrids for their cold germination phenotype. ^b Accession number of the gene or EST in GenBank, SwissProt/TrEMBL, or PIR databases. ^c Percent identity between the query sequence of the cDNA fragment and the sequence found in the databases. ^d Length (no. of nucleotides) of the cDNA fragment. ^e Functional category based on published literature or Kyoto Encyclopedia of Genes and Genomes database. ^f Description of gene or the source tissue of cDNA library for the EST.

Table II. Differentially expressed genes for the DT trait revealed by mRNA profiling

The expression levels of mRNA extracted from R5 stage embryos at 40% kernel moisture content were compared between the F₁ hybrids that were desiccation tolerant (M0021-syn4 × M0337-syn4) and desiccation sensitive (M0337-syn4 × M0021-syn4). The gene/maize EST identities of the cDNA fragments were obtained by BLAST search of the GenBank and SwisProt/TrEMBL databases with probability value of $\leq 1.0 \times 10^{-7}$.

Change ^a	Accession No. ^b	Percent Identity ^c	cDNA Size ^d	Function ^e	Gene Description or Source of EST ^f
<i>-fold</i>			<i>nt</i>		
-1.7	P23901; AW231664	89; 96	357	Carbohydrate and glycerolipid metabolism	Aldose reductase (EC 1.1.1.21); early embryo cDNA library
1.9	U39958	99	322	Carbohydrate metabolism	NADP-malic enzyme root isoform
1.7	M58656; AW225169	92; 100	84	Carbohydrate metabolism	Pyruvate orthophosphate dikinase; early embryo cDNA library
1.7	AAF86353	97	295	Cell growth and signaling	SerThr protein phosphatase
1.9	P23903	100	293	Cell wall/defense related	β -1,3-glucanase A1 precursor
1.7	AL033545	93	155	Cell wall metabolism	Extensin-like protein
-1.8	AC007170	78	122	Citrate cycle/glyoxylate metabolism	Aconitate hydratase (cytoplasmic)
1.6	AF053468	99	182	Cochaperones	DnaJ-related protein
1.6	AF085279	100	270	Cytoskeletal	Hypothetical ankyrin-like protein
2.2	L34693	100	105	Defense related (PR-5)	Thumatin-like protein
2.1	X69960	99	114	DNA synthesis and replication	H2B histone
1.7	M36659	97	89	DNA synthesis and replication	Histone H4 (H4C13) gene
1.6	X57312	100	239	DNA synthesis and replication	H2B histone
1.6	P80269; AW585278	90; 98	260	Electron transport and oxidative phosphorylation	NADH-ubiquinone oxidoreductase (EC 1.6.5.3 and 1.6.99.3); mixed adult tissues cDNA library
1.6	AF004210	99	149	Fatty acid/C21 steroid metabolism	Cytochrome P-450
1.6	Q9XGN7	95	298	Glyoxylate and dicarboxylate metabolism	Citrate synthase (EC 4.1.3.7)
1.8	Y09506	99	281	mRNA processing	Transformer-SR ribonucleoprotein
8	P81008	100	125	Plant defensins (Na ⁺ 2 channel blocker)	γ -ZEATHIONIN 1
1.8	AAB31685	98	96	Protease inhibitor	Trypsin inhibitor
1.8	AF026977	99	194	Protect against oxidative damage	Microsomal glutathione S-transferase 3
1.7	AF026977	100	140	Protect against oxidative damage	Microsomal glutathione S-transferase 3
1.5	AF107837	94	217	Protein degradation	26S proteasome subunit
-1.6	BAA96834; BE518965	88; 98	332	Protein degradation	β 1-subunit of 20S proteasome; tassel primordium cDNA library
-2	Q41365	100	43	Protein degradation/signaling	Protease regulatory subunit
6.8	M12145	98	136	Protein destination and storage	Maize 19-kD zein
1.8	X56117	100	103	Protein destination and storage	Maize 27-kD zein locus DNA
1.7	AF031569	100	327	Protein destination and storage	22-kD α -Zein gene cluster
1.7	P09189	99	301	Protein folding and stability	Heat shock cognate 70-kD protein
1.7	Q05045; A1712268	71; 99	149	Protein folding and stability	Heat shock protein 60 kD (CPN60-1, mitochondrial); endosperm
-1.5	BAB09366; AW076363	70; 100	101	Protein processing and degradation	Aspartyl protease; root cDNA library
-1.5	U92045	99	159	Protein synthesis	Ribosomal protein S6 RPS6-1 (maize)
-1.7	AJ237573	98	172	Retrotransposon	gag polyprotein precursor
-1.9	U70855	93	132	RNA/protein synthesis	Tyrosyl-tRNA synthetase
1.9	P25070	100	58	Signaling	Calmodulin-related protein (touch induced)
-1.5	P52409; BE056116	81; 100	362	Starch and Suc metabolism	Glucan endo-1,3- β -glucosidase (endohydrolyase) (EC 3.2.1.39); mixed adult tissues cDNA library
1.7	P04713	98	276	Starch metabolism	Granule-bound glycogen (starch) synthase precursor (EC 2.4.1.11)
1.6	AF079260	100	86	Starch metabolism	Granule-bound starch synthase (waxy)
2.4	AL035356	100	235	Sulfur metabolism	Putative alliinase
3.3	AC005313	100	145	Transcription factor	Chloroplast nucleoid DNA-binding protein
2	AF087672	100	92	Transcription factor	eRFS (<i>Mus musculus</i>)
-2	AC006533	98	90	Transcription factor	Putative ARI, RING finger protein
1.7	CAB90214; AW288485	88; 100	295	Translation machinery	Elongation factor 1- β ; fusarium infected silk cDNA library

(Table continues.)

Table II. (Table continued from previous page.)

Change ^a	Accession No. ^b	Percent Identity ^c	cDNA Size ^d	Function ^e	Gene Description or Source of EST ^f
-fold			nt		
3.5	AI711766	92	198	Unclassified	Endosperm cDNA library
3	AI739952	99	209	Unclassified	Endosperm cDNA library
2.3	U84881	99	429	Unclassified	Envelope glycoprotein
2	AW927832	100	89	Unclassified	Mixed adult tissues cDNA library
1.9	AI619108	100	388	Unclassified	Leaf primordia cDNA library
1.8	AI740005	100	310	Unclassified	Endosperm cDNA library
1.8	AF081810	97	305	Unclassified	LdOrf-129 peptide (<i>Lymantria dispar</i> nucleopolyhedrovirus)
1.7	D15843; AW225169	90; 100	265	Unclassified	Early nodulin; early embryo cDNA library
1.7	Q9XIR5; AI670584	72; 100	133	Unclassified	Translational activator; endosperm cDNA library
1.6	AW066498	100	170	Unclassified	Mixed stages of another and pollen cDNA library
1.6	AW202549	99	282	Unclassified	Early embryo cDNA library
1.6	AI600894	100	135	Unclassified	Leaf primordia cDNA library
1.5	AC005850	100	113	Unclassified	Unknown protein (Arabidopsis)
1.5	AI902013	99	353	Unclassified	Inbred Tassel cDNA library
1.5	AF049892	99	171	Unclassified	Embryo-specific protein
-1.5	AC004218	100	65	Unclassified	Unknown protein (Arabidopsis)
-1.6	AI920497	99	326	Unclassified	Root cDNA library
-1.6	AL035356	99	278	Unclassified	Putative protein (Arabidopsis)
-1.9	O22155	83	267	Unclassified	T14P1.23 PROTEIN—Arabidopsis (Mouse-ear cross)

^a The values, positive and negative, represent fold changes of mRNA abundance in the tolerant versus sensitive reciprocal F₁ hybrids to high temperature desiccation. ^b Accession number of the gene or EST in GenBank, SwissProt/TrEMBL, or PIR databases. ^c Percent identity between the query sequence of the cDNA fragment and the sequence found in the databases. ^d Length (no. of nucleotides) of the cDNA fragment. ^e Functional category based on published literature or Kyoto Encyclopedia of Genes and Genomes database. ^f Description of gene or the source tissue of cDNA library for the EST.

(by GeneCalling) or cloned sequence identification. Confirmation was revealed by the selective disappearance of the corresponding peak in the electrophoretic scans (Fig. 5).

Protein Expression Profiles

The average protein yields were 14% and 19.5% (dry weight basis) for embryos at 40% moisture content and 11-d-old germinating embryo axes, respectively, suggesting that most of the proteins were extracted from the tissue. The principle steps involved in the protein expression profiling are illustrated in Figure 6.

A total of 7,419 and 6,585 unique features (proteins with different pI/M_r combinations) were detected across samples within the CG and DT traits, respectively. Of these, 2,641 and 1,876 features were consistently detected above the background threshold level for CG and DT experiments, respectively. The F-test identified 117 features in CG and 205 features in DT samples that were significantly differentially expressed ($P < 0.1$) with ≥ 1.5 -fold change between the reciprocal F₁s. A subset of these features was further analyzed to obtain the protein identification.

The selected protein features were excised from

the gels and subjected to in-gel trypsin digestion. The database search using tryptic-oligopeptide mass and tandem mass spectral (MS/MS) information with SEQUEST program (Fennigan Corp.) resulted in the identification (annotation) of many proteins. The representative PEMs with the annotated proteins that are differentially expressed between the reciprocal F₁ hybrids are shown in Figure 7. The reason why some of the protein features are difficult to see on the gels is that they are expressed at low levels and can only be detectable by the highly sensitive scanners.

Protein identifications obtained for 56 features in the CG experiment (Fig. 7A) and 54 features in the DT experiment (Fig. 7B) are listed in Tables III and IV, respectively. Identification of the remaining features in each trait could not be obtained because of the database limitations. The oligopeptide masses and MS/MS spectral data of some of the features matched more than one gene. For example, globulin-2 precursor and nucleoside diphosphate kinase I with pI 6.5 and M_r 12,010 (protein feature no. 4,413; Table III) or globulin 1L and superoxide dismutase with pI 4.9 and M_r 13,670 (protein feature no. 3,544; Table IV) had either the same or different oligopeptide sequence matches. On the other hand,

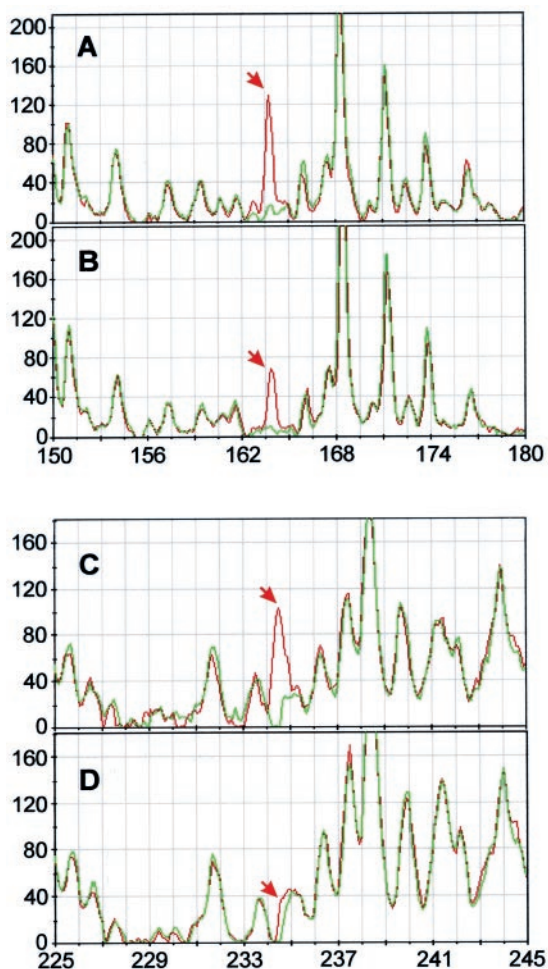


Figure 5. Competitive PCR confirmation of the differentially expressed mRNA between the reciprocal F_1 hybrids. The traces in red are controls and those in green are of competitive PCR using gene specific primers (see “Materials and Methods” for details). A and B show traces of cDNA fragments of the reciprocal F_1 hybrids that were high (M0081-syn0 \times M0023-syn0) and low (M0023-syn0 \times M0081-syn0), respectively, for the CG phenotype. Competitive PCR with specific primers to abscisic acid- and stress-inducible protein (AF09573; see Table I) caused the red (control) peak (shown by the red arrow) to disappear, confirming the gene identity. Similarly, C and D show the traces of the desiccation tolerant (M0021-syn4 \times M0337-syn4) and sensitive (M0337-syn4 \times M0021-syn4) reciprocal F_1 hybrids, respectively, showing the competitive PCR confirmation with alliinase (AL035356; see Table II) specific primers. The red traces in each of the panels represent an average of six electrophoretic traces of the digests from replicate samples. The differential expression (i.e. fold change) of the cDNA fragments can be gauged by comparing the peak heights of the red traces between A and B and also between C and D. The length of the cDNA fragment is shown along the x axis as number of nucleotides, and the height of the peaks (y axis) represent the abundance in arbitrary fluorescence units.

two or more protein features had the same annotation. For example, several protein features in both CG (Table III) and DT (Table IV) were annotated to be globulin-2 precursor proteins.

DISCUSSION

Screening and Phenotypic Analysis

Significant variation observed for CG and DT in this population of RILs enabled us to select the most

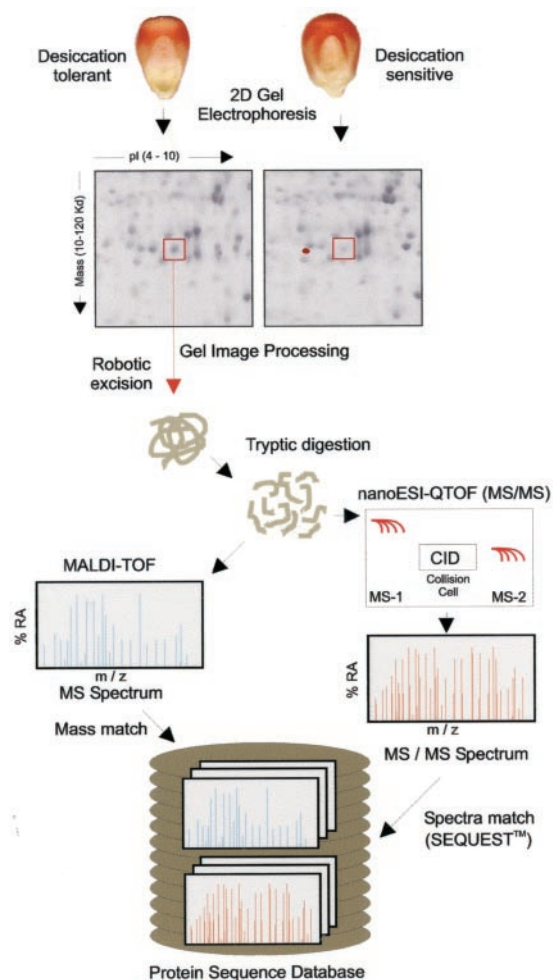


Figure 6. Schematic representation of the protein profiling process. Protein extracts from the samples were resolved on two-dimensional gels, first by their pI followed by their relative molecular mass. The gels were stained with a highly sensitive fluorescent dye and were scanned for image data processing. Each protein feature on the gel was cataloged by its position (pI and M_r) and density (volume) in the gel, referred to as PEM. The PEMs of replicate samples of each F_1 hybrid were compiled and the differentially expressed protein features between the reciprocal F_1 hybrids were identified. These protein features were then robotically excised and subjected to trypsin digestion (site-specific cleavage at the C-terminal of Lys or Arg residue), and masses of the resulting oligopeptides were measured using matrix-assisted laser-desorption/ionization time of flight mass spectrometer (MS). A portion of the digest was also subjected to MS/MS analysis using nano-electrospray ionization source (Z-spray) on a quadrupole time of flight MS. The uninterpreted fragmentation spectra in combination with the masses of the tryptic fragments were used to query the databases (GenPept, SwissProt, and Pioneer/DuPont and Oxford GlycoSciences’ proprietary) using a computer program (SEQUEST program, Fennigan Corp., San Jose, CA) to obtain the protein identification (for details, see “Materials and Methods”).

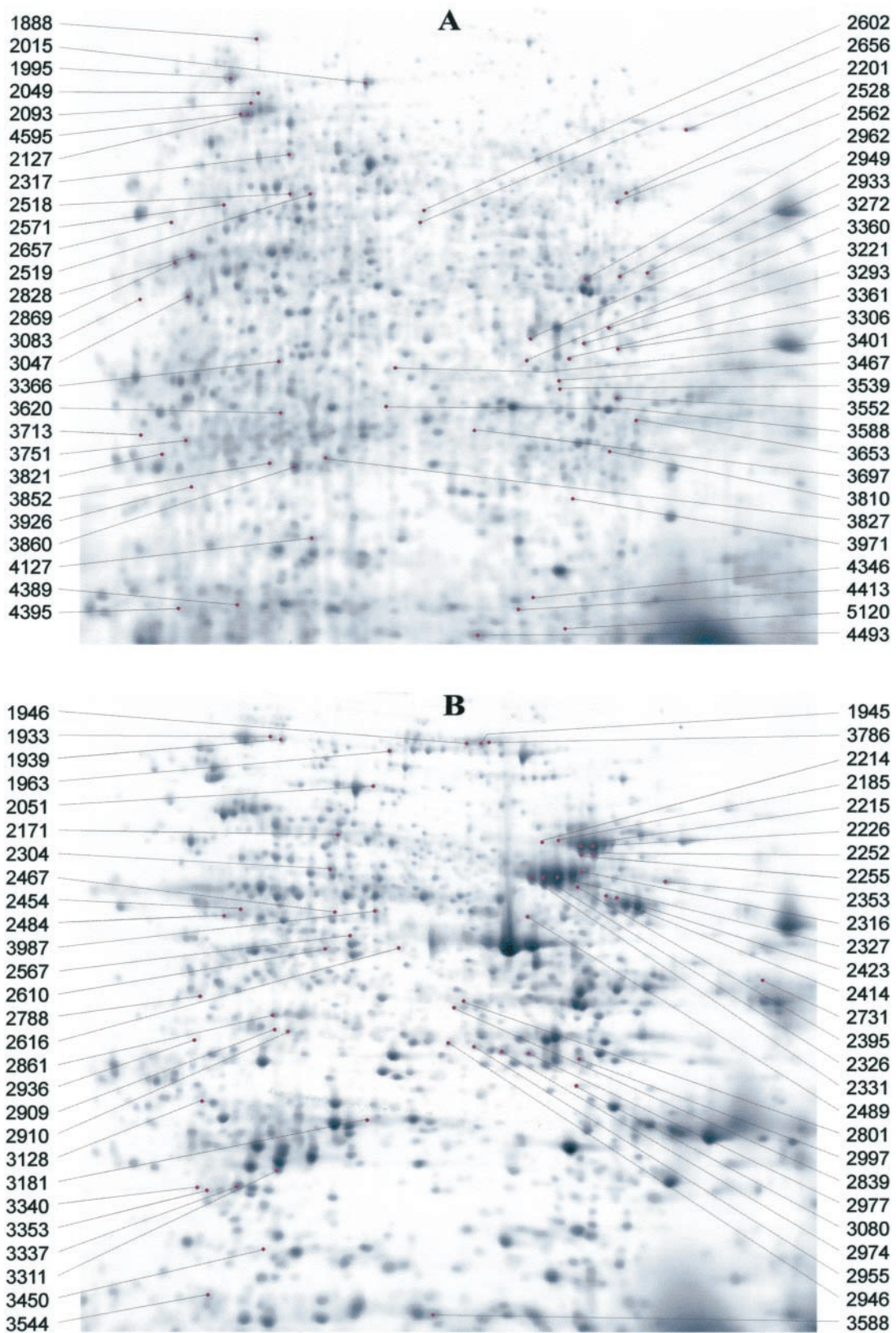


Figure 7. PEMs of representative samples for the CG (A) and DT (B) experiments with annotations. The protein features indicated by red spots were differentially expressed between the reciprocal F_1 hybrids. The identification of each of the proteins and their descriptions are provided in Tables III and IV for A and B, respectively.

Table III. Differentially expressed genes for the CG trait revealed by protein expression profiling

The expression levels of proteins extracted from embryo axes tissue of seeds germinated for 11 d at 12.5°C in the dark were compared between the reciprocal F₁ hybrids that were high (M0081-syn0 × M0023-syn0) and low (M0023-syn0 × M0081-syn0) for CG phenotype. The gene identities of the proteins were obtained by matching the mass sizes of the trypsin-derived oligopeptides as well as the uninterpreted tandem mass spectra to those generated by a computer algorithm (SEQUEST, Fennigan Corp) from the GenPept, SwisProt, or proprietary databases.

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
	<i>-fold</i>							<i>n</i>
5120	2.92	Ropane alkaloid biosynthesis	Putative tropinone reductase	Q9ZW03	6.8	9383	GIGQAVVEELAR	1
4395	1.88	Glycolysis/gluconeogenesis	Triosephosphate isomerase 1 (maize; EC 5.3.1.1)	L00371	4.7	10220	TNASPEVAESTR	3
4493	-4.84	Cold/stress responsive with unknown function	LEA protein (EMB564)	P46517	6.2	10610	SVEAQEHLAEGR	6
4413	-2.08	Phosphorylation of nucleoside diphosphates and proteins	Nucleoside diphosphate kinase 1	Q02254	6.5	12010	IIGATNPLASEPGTIR	3
4413	-2.08	Seed storage protein	Globulin-2 precursor	S15675	6.5	12010	LLAFGADEEQVDR	7
4389	1.58	Translational regulation	Elongation factor 2	P13060	5.0	12310	SQILSQEFGWDK; DSVVAGFQWASK	7
4346	1.66	Ubiquitin-mediated protein degradation	Ubiquitin-conjugating enzyme E2 (Catharanthus roseus)	AF091621	6.6	12700	LELFLPEEYPMMAAPK	3
4127	2.19	Unknown	Gly-rich protein	P27484	5.4	16180	SLNDGDAVEYTVGSGNDGR	5
3971	2.15	Ca(2+) and phospholipid-binding proteins with unknown function	Annexin p33	CAA66900	6.9	19660	SITDEISGDFER	5
3926	2.55	Methionine metabolism	5-Methyltetrahydropteroyl-triglutamate-homocysteine S-methyltransferase (EC 2.1.1.14)	Q9LM03	4.7	20680	IPSAEELADR	3
3860	-1.83	Seed storage protein	Globulin-2 precursor	S15675	5.3	21650	EGEGVIVLLR; FTHELLEDAVGNRY; GEITASEEQIR; AFLQPSHYDADEVMFVK	7
3827	-2.62	Seed storage protein	Globulin-2 precursor	S15675	5.5	22000	VAELEAAPR; GEITASEEQIR; EGEGVIVLLR; FTHELLEDAVGNRY	6
3852	-1.87	Seed storage protein	Globulin-2 precursor	S15675	5.1	22040	GEITASEEQIR	7
3810	-1.69	Signaling (unknown in plants)	Cytokeratin type II	NP_004684	7.4	22060	AEAESWYQTK	1
3821	1.62	Fru metabolism	Fructokinase	AAA80675	4.6	23480	APGGAPANVAIVSR; TQILSIWDQADIVK	7
3751	1.55	Microtubule-associated protein	β-3 and -6 tubulin (maize)	L10633	4.7	24340	YTGNSDLQLER; AVLMDLEPGTMDSVR	4
3713	2.13	Microtubule-associated protein	DYNACTIN, 150-kD isoform	Q14203	4.5	24770	LHDAADEIESLR; LGDQLQTAYQEK	6
3653	-2.55	Seed storage protein	Globulin-2 precursor	S15675	7.7	24900	LLAFGADEEQVDR	8
3697	2.49	Chaperonin	Cytosolic chaperonin, δ-subunit	CAA09989	6.2	25610	GSNQLVIDEAEER	4
3620	1.87	Nucleotide sugar and aminosugar metabolism and glycosaminoglycan degradation	β-Glucosidase, root meristem (EC 3.2.1.-) precursor	A48860	5.2	26850	SIVEDYTYFAK	6
3552	-1.55	Unknown	Hypothetical protein	BAB21159	7.4	27660	IFDSLPAEEQR	7
3588	-1.54	Pyruvate metabolism/cellular detoxification	Glyoxalase II (cytoplasmic isozyme, EC 3.1.2.6)	O24496	5.7	28020	FSVNSPVEAMR; FSVNSPVEAMR; GHISYYVTSK	6

(Table continues.)

Table III. (Table continued from previous page.)

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
	<i>-fold</i>							<i>n</i>
3539	2.06	Disease response gene act as membrane-bound chaperone to stabilize mitochondrial proteins	Prohibitin (maize)	AF236369	6.8	28660	LISEATAMAGTGLIELR; SPNVAYIPSGENGK; DLQMVNLTLR	9
3401	-4.95	Pyruvate metabolism/cellular detoxification	Glyoxalase I (EC 4.4.1.5)	AB017042	5.8	30870	VVLVDNTDFLK; YTIAMLGVADEDK	9
3467	-2.11	Disease response gene act as membrane-bound chaperone to stabilize mitochondrial proteins	Prohibitin (maize)	AF236369	6.8	30890	DLQMVNLTLR; SPNVAYIPSGENGK; VLPSIGNEVLK	10
3361	-2.21	Pentose and glucuronate interconversions/pyruvate metabolism	Aldose reductase (aldehyde reductase; EC 1.1.1.21)	P23901	6.9	31860	NLAHDPLVEK	10
3306	-1.68	Ca ²⁺ and phospholipid-binding proteins with unknown function	Annexin p33	CAA66900	7.4	32010	SITDEISGDFER; ADPQDEYLR	14
3366	-3.02	Cold/stress responsive with unknown function	LEA protein (LEA D-34)	P09444	5.2	32160	FGDVLVDSGELADQPVAPR; LQAEEQSVLGGTQK	7
3360	-2.68	Seed storage protein	Globulin-2 precursor	S15675	6.5	32170	EGEGVIVLLR	9
3293	-2.51	Pentose and glucuronate interconversions/pyruvate metabolism	Aldose reductase (aldehyde reductase; EC 1.1.1.21)	P23901	7.1	33210	NLAHDPLVEK	12
3272	13.91	Protein processing and degradation	Trypsinogen 7 (<i>M. musculus</i>)	AAB69044	6.5	34380	YVNWIIQQTIAAN	0
3221	-1.96	Cold and ABA responsive with probable adaptive function	osr40g2	T03962	7.4	34400	LNFDFAFHGDK; LNFDFAFHGDK; DEEGYPAFALVNK	12
3047	1.62	Chaparonin	Heat shock protein 82	P33126	4.7	39550	DDQLEYLEER; SDLVNNLGTIAR	14
3083	3.34	Protein processing and degradation	Cysteine proteinase Mir3 (maize)	AF019147	4.5	39820	AVANQPISVAIEAGGR	1
2962	-1.94	Cold/stress responsive with unknown function	LEA protein in group 3	D64140	7.1	40220	LTLGQQGHVDVR	5
2949	-2.38	Glycolysis/gluconeogenesis	Fru-bisphosphate aldolase, cytoplasmic isozyme (EC 4.1.2.13)	M16220	7.5	40490	LSSINVENVEENR; GDAAADTESLHVK	14
2933	-2.67	Glycolysis/gluconeogenesis	Aldolase (maize) (EC 4.1.2.13)	M16220	7.9	40690	VTPEVIAEYTVR; LSSINVENVEENR	8
2869	1.61	Chaparon/protein trafficking in ER—stress inducible	78-kD Glc-regulated protein precursor (GRP 78)	Q16956	4.6	44210	FEELNMDLFR	2
2869	1.61	Chaparonin	Heat shock cognate 70-kD protein	P09189	4.6	44210	FEELNMDLFR	6
2828	1.54	Chaparonin	Heat shock protein 70	P11143	4.7	45410	FEELNMDLFR; SSVHDVVLVGGSTR	8
2656	1.82	Starch and Suc metabolism and flavonoids, stilbene and lignin biosynthesis	β -Glucosidase, chloroplast precursor (maize; EC 3.2.1.21)	U33816	5.9	47390	IGLAFDVMGR; SIVEDYTYFAK	12
2657	1.60	Nucleotide-excision repair protein that inhibits multi-ubiquitin chain formation and the degradation of proteolytic substrates	RAD23 protein	Q9STA6	4.6	47580	LIQENQAEFLR; IIETTQQGSTYR; GTNFEIASPASVADVK	7
2602	-1.54	Retroelement	Putative retroelement pol polyprotein (Arabidopsis)	AAD23707	5.9	48790	WVEAIASPTNDAK	3

(Table continues.)

Table III. (Table continued from previous page.)

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
	<i>-fold</i>							<i>n</i>
2562	-1.66	Gly, Ser and Thr metabolism	Hydroxymethyltransferase (BC 2.1.2.1)	O23254	7.5	49710	YSEGMPGAR	10
2571	-3.12	Microtubule-associated protein	Tubulin β -1 and -5 chain	P18025/ Q43697	4.9	50630	FPGQLNSDLR; VSEQFTAMFR; AVLMDLEPGTMDSVR; AVLMDLEPGTMDSVR; YGGDSDLQLER; NSSYFVEWIPNNVK; ALTVPELTQQMWDAK	7
2528	-2.61	Seed storage protein	Globulin precursor (maize)	M24845	7.6	51060	VFLAGADNVLQK	15
2518	-1.98	Glycolysis/gluconeogenesis Phe, Tyr, and Trp biosynthesis	Enolase 1 (EC 4.2.1.11)	P26301	5.3	51430	VNQIGSVTSEIAVR; VQIVGDDLLVTNPTR; GNPTVEVDVGLSDGSYAR	18
2519	-1.58	Glycolysis/gluconeogenesis Phe, Tyr and Trp biosynthesis	Enolase 1 (EC 4.2.1.11)	P26301	5.4	52110	VNQIGSVTSEIAVR; VQIVGDDLLVTNPTR	9
2317	-1.82	Chaparonin	Chaperonin 60 (maize)	L21007	5.3	57220	NVVIEQSFQAPK; LLEQENTDLGYDAK	5
2201	-2.84	Unknown	Putative protein	AL080282	8.5	63670	GIVAGEEELVPVGGK	3
2127	-1.81	Chaparonin	Heat shock protein 70	P11143	5.0	67510	NAVVTVPAYFNDSQR; TTPSYVAFTDSEK; ATAGDTHLGGEDFDNR; MVNHVFVQEFK; EIAEAYLGSTIK; VEIANDQGNR; SSVHDVVLVGGSTR	9
4595	-1.51	Chaparonin	Heat shock 70-kD protein	P11143	5.0	67590	EIAEAYLGSTIK; NAVVTVPAYFNDSQR; FEELNMDLFR	10
2093	2.85	Chaparonin	Heat shock protein 70	P26413	5.0	71950	NAVVTVPAYFNDSQR; DAGVIAGLNVLR; EIAEAYLGSTIK	9
2049	-2.19	Chaparon/protein trafficking in ER	Lumenal binding protein cBiPe2 (maize)	U58208	5.1	77220	FEELNNDLFR; INDAVVTVPAYFNDAQR	14
2015	-1.63	Methionine metabolism	5-Methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase (EC 2.1.1.14)	Q9LM03	5.7	77660	YGAGIGPGVYDIHSPR; YLFAGVVVDR; IPSAEEIADR; GTQTLGLVTSAGFPAGK; ISEEEYVTAIK; FALESFWDGK	16
1995	-2.07	Chaparonin	Heat shock protein 82	P33126	5.0	81110	GIVDSEDLPLNISR	25
1888	-4.37	Cell division	Cell division cycle protein 48 homolog	Q96372	5.1	103100	TALGTSNPSALR; LDEVGYDDVGGVR	17
1888	-4.37	Chaparon targeting ubiquitinated proteins to proteosomes for degradation	Valosin-containing protein (soybean [Glycine max])	U20213	5.1	103100	KGDLFLVR; LDEVGYDDVGGVR	17

^a Unique identification number assigned to a protein spot based on its isoelectric point and molecular mass across all the gels in an experiment. ^b The values, positive and negative, represent fold changes of protein abundance in high vs. low reciprocal F_1 hybrids for their CG phenotype. ^c Functional category based on published literature or Kyoto Encyclopedia of Genes and Genomes database. ^d Accession number of the gene in GenBank, SwissProt/TrEMBL, or PIR databases. ^e pI of the protein. ^f Relative molecular mass (daltons) of the protein. ^g Sequences of the oligopeptides derived from complete peptide (gene) sequence in the databases whose ionization mass spectra were consistent with the γ -type fragmentation spectra observed in the sample. ^h Number of trypsin-derived oligopeptide masses that matched the gene sequence.

Table IV. Differentially expressed genes for the DT trait revealed by protein expression profiling

The expression levels of proteins extracted from R5 stage embryos at 40% kernel moisture content were compared between the F₁ hybrids that were desiccation tolerant (M0021-syn4 × M0337-syn4) and desiccation sensitive (M0337-syn4 × M0021-syn4). The gene identities of the proteins were obtained by matching the mass sizes of the trypsin-derived oligopeptides as well as the uninterpreted tandem mass spectra to those generated by a computer algorithm (SEQUEST, Fennigan Corp.) from the GenPept, SwissProt, or proprietary databases.

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
	<i>-fold</i>							<i>n</i>
3544	2.40	Antioxidant	Superoxide dismutase, chloroplast [Cu-Zn] precursor	P93407	4.9	13670	GGHELSTLSTGNAGGR	3
2454	-3.89	Chaparonin	HSP-associated protein-like	O49648	5.1	49890	IPAAAPSFESPK	7
3786	15.23	Chaparonin/caseolytic protease B	Heat shock protein HSP101 (maize)	AF133840	6.5	105400	ALAEQLFDDENLLVR; VQLDSQPPEIDNLER	14
1945	2.78	Chaparonin/caseolytic protease B	Heat shock protein HSP101 (maize)	AF133840	6.4	111500	VQLDSQPPEIDNLER; WTGIPVTR	37
1946	-7.02	Chaparonin/caseolytic protease B	Heat shock protein HSP101 (maize)	AF133840	6.3	117000	VQLDSQPPEIDNLER	13
2801	3.57	Citrate cycle/Glyoxylate and dicarboxylate metabolism	Malate dehydrogenase (soybean)	AF180335	6.3	39290	NGVEEVLGLGELNEFEK	5
2788	3.82	Cold/stress responsive with unknown function	LEA-like protein	O80576	4.9	39880	DFGSVAVWDMIR; IDVDTPFGNMK	7
2610	2.73	DNA methylation	O-Methyltransferase ZRP4 (EC 2.1.1.-)	P47917	5.5	45380	VIIIDTVLGSR	4
3080	2.80	Fatty acid biosynthesis	3-Oxoacyl-[acyl-carrier protein] reductase precursor (EC 1.1.1.100)	P28643	7.1	29890	LEAPVVVVVTGASR	3
1963	5.32	Glycolysis	Pyruvate, orthophosphate dikinase (maize; EC 2.7.9.1)	J03901	5.8	94460	FAYDSFR	7
1939	3.79	Glycolysis	Pyruvate, orthophosphate dikinase (maize)	M58656	5.4	104000	SDFEGIFR; EMQDIEFTVQENR	17
1933	2.27	Glycolysis	Pyruvate, orthophosphate dikinase (maize)	M58656	5.3	107200	FAYDSFR	22
2051	2.41	Met metabolism	5-Methyltetrahydropteroyl-triglutamate—homocysteine S-methyltransferase (EC 2.1.1.14)	Q9LM03	5.8	74300	YLFAGVVDGR; IPSAEEIADR; FALESFWDGK	11
2484	-40.08	Microtubule-associated protein	Tubulin α-1	P14640	5.0	49280	AVFVDLEPTVIDEVR; TIGGGDDAFNTFFSET-GAGK	9
2997	7.34	Pentose and glucuronate interconversions	Aldose reductase (EC 1.1.1.21)	P23901	7.1	33240	NLAHIDLVEK; SGHTIPAVGLGTWR	12
3181	2.09	Protein degradation	Multicatalytic endopeptidase	CAA73624	5.7	25020	TTIFSEGR	3
3588	2.15	Seed storage protein	Globulin-2 precursor	S15675	6.1	12050	LLAFGADEEQQVDR	2
3544	2.40	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	4.9	13670	VFLAGADNVLQK	6
3353	-2.03	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	4.9	19840	VFLAGADNVLQK	5
3340	-9.32	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	4.9	20150	VFLAGADNVLQK	5
3337	-2.94	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	5.1	20260	VFLAGADNVLQK	5
3311	3.11	Seed storage protein	Globulin-2 precursor	S15675	5.4	21090	FTHELLEDAVGNRYR; GEITTASEEQIR; VVMLLSPVVSTSGR	7
2977	4.64	Seed storage protein	Globulin-2 precursor	S15675	6.7	32910	VVMLLSPVVSTSGR; GEITTASEEQIR	8

(Table continues.)

Table IV. (Table continued from previous page.)

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
	<i>-fold</i>							<i>n</i>
2974	3.31	Seed storage protein	Globulin-2 precursor	S15675	6.5	32960	GEITTASEEQIR; LLDMDVGLANIAR	11
2955	3.09	Seed storage protein	Globulin-2 precursor	S15675	6.4	33500	FTHELLEDAVGNYR; GEITTASEEQIR; LLDMDVGLANIAR	8
2946	2.34	Seed storage protein	Globulin-2 precursor	S15675	6.2	33570	GEITTASEEQIR; LLDMDVGLANIAR	8
2910	-2.59	Seed storage protein	Globulin-1S, GLB1S	A53234	5.4	35200	VFLAGADNVLQK; IAYVPGNK	8
2909	-2.21	Seed storage protein	Globulin-2 precursor	S15675	5.3	36060	GEITTASEEQIR	6
2731	-2.31	Seed storage protein	Globulin-2 precursor	S15675	9.9	42390	LLAFGADEEQVDR	11
2567	2.01	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	5.6	46700	VAVLEANPR; VLRPFDEVSR	7
2489	2.07	Seed storage protein	12s Globulin	P14812	6.7	49730	LDQADVSPGAGR; VVVDAMGLLLPR	8
2414	3.12	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	7.3	51910	VAVLEANPR	9
2423	3.88	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	7.4	52140	VFLAGADNVLQK	12
2395	-3.52	Seed storage protein	Globulin-1S, GLB1S	A53234	7.1	54060	RPYVFDR; VFLAGADNVLQK; VAVLEANPR	12
2327	-2.16	Seed storage protein	Globulin-1S, GLB1S	A53234	7.0	55070	RPYVFDR; VAVLEANPR; NPESFLSSFSK	13
2353	-3.27	Seed storage protein	Globulin-1S, GLB1S	A53234	8.2	55320	VAVLEANPR; VFLAGADNVLQK	12
2331	-3.00	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	6.7	55450	VLRPFDEVSR; VAVLEANPR	10
2326	-3.78	Seed storage protein	Globulin-1S, GLB1S	A53234	6.9	56070	VLRPFDEVSR; VAVLEANPR	17
2316	-3.12	Seed storage protein	Globulin-1S, GLB1S	A53234	7.1	56290	VAVLEANPR; VFLAGADNVLQK	16
2255	-4.07	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	7.2	57650	VAVLEANPR	21
2252	-4.52	Seed storage protein	Globulin-1S, GLB1S	A53234	7.3	58310	RPYVFDR; VAVLEANPR	18
2226	-3.10	Seed storage protein	Globulin-1S, GLB1S	A53234	7.2	59830	RPYVFDR; VAVLEANPR	18
2215	-4.52	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	7.2	60430	RPYVFDR	20
2185	-2.63	Seed storage protein	Vicilin-like storage protein Glb1-L, embryo	B53234	7.0	61790	VAVLEANPR; VFLAGADNVLQK	18
2171	-36.20	Seed storage protein	Globulin-1S, GLB1S	A53234	5.6	62040	RPYVFDR; VAVLEANPR; VFLAGADNVLQK	15
2214	-8.67	Seed storage protein	Globulin-1S, GLB1S	A53234	6.8	62320	VAVLEANPR	13
2304	2.34	Starch biosynthesis	Amyloplast-specific transit protein (maize)	M24258	5.6	55630	FAFSDYPELNLPER	13
2304	2.34	Starch biosynthesis	Granule-bound glycogen (starch) synthase precursor (EC 2.4.1.11)	P04713	5.6	55630	FAFSDYPELNLPER; YGPDAGTDYR	6
2861	-4.76	Translational machinery	60S acidic ribosomal protein P0	AF227622	5.4	37080	GTVEITPVELIK; VLIALADNVGSK	4
3450	22.81	Translational machinery/signaling	Translation initiation factor 5A (maize)	AAB88614	5.2	16090	LPTDETLVAQIK	2
2467	2.68	Translational terminator	Eukaryotic release factor 1 homolog	Q39097	5.6	49530	QSVLAAITSAQQR	5
3128	2.12	Unknown	Unknown protein (Arabidopsis)	AC006283	4.9	27870	TINEEEGSPIDQLFFR	2

(Table continues.)

Table IV. (Table continued from previous page.)

Protein Feature No. ^a	Change ^b	Function ^c	Protein Name	Accession No. ^d	pI ^e	Mass ^f	Sequence Match ^g	Mass Matches ^h
2936	-fold 3.40	Unknown	CG4527 gene product (fruitfly [<i>Drosophila melanogaster</i>])	AE003464	4.9	34470	KEEDMVK	<i>n</i> 7
2839	2.10	Unknown	Unknown protein (Arabidopsis)	AC007171	6.2	38490	IEGGSFLIVPR	5
2616	2.57	Unknown	Unknown protein	AC005824	5.9	45420	MDFIPGVDGPSEGVP	4
3987	2.89	Unknown	Unknown protein (Arabidopsis)	AC007017	5.8	50170	FDLWESEGDVR; LFITQTGSDSWVGR	4

^a Unique identification number assigned to a protein spot based on its pI and molecular mass across all the gels in an experiment. ^b The values, positive and negative, represent fold changes of protein abundance in the tolerant versus sensitive reciprocal F₁ hybrids to high temperature desiccation. ^c Functional category based on published literature or Kyoto Encyclopedia of Genes and Genomes database. ^d Accession number of the gene in GenBank, SwissProt/TrEMBL, or PIR databases. ^e pI of the protein. ^f Relative molecular mass (daltons) of the protein. ^g Sequences of the oligopeptides derived from complete peptide (gene) sequence in the databases whose ionization mass spectra were consistent with the γ -type fragmentation spectra observed in the sample. ^h Number of trypsin-derived oligopeptide masses that matched the gene sequence.

divergent inbreds for evaluation of parent-of-origin effect in reciprocal F₁ hybrids. Because the RIL population was derived from a single cross (B73 × M017), only two possible alleles can occur for any gene at a given locus. Furthermore, each RIL was self-pollinated for at least 11 generations, creating a population of inbreds each of which is homogeneous (one haplotype) and homozygous at most of the loci (Burr and Burr, 1991). Therefore, this RIL population was ideal for studying the genetics of many traits, especially complex ones such as CG and DT included in this study. Comparison of reciprocal F₁ hybrids produced by crossing divergent RILs provided an ideal system (such as comparison of isogenic lines) to identify genes that influence the phenotypes considered.

Of the 25 RIL combinations (five high × five low) in each trait, the majority did not show significant differences between their reciprocal F₁ hybrids (data not shown). However, reciprocal F₁ hybrids between specific divergent RILs showed significant phenotypic differences, suggesting that parent-of-origin effects were influencing these traits in a genotype-specific manner. Differential phenotypic expression between reciprocal F₁ hybrids, as was shown in other studies, is considered primary evidence of the imprinting phenomenon in plants (Kermicle, 1970; Lin, 1982; Ohad et al., 1996; Chadhury et al., 1997; Grossniklaus et al., 1998). Pinnell (1949) and Bdllya and Burris (1988) reported phenotypic differences between the reciprocal F₁ hybrids of selected maize genotypes for CG and DT traits, respectively, but the underlying molecular bases were not explored.

Cytoplasmic and Dosage Effects

It is not uncommon to see phenotypic differences between reciprocal F₁ hybrids for many morphological and agronomic traits in maize, especially those caused by cytoplasmic factors (mitochondrial and

chloroplast genomes, commonly referred to as the maternal effect). To discern whether such factors were involved, reciprocal F₁ hybrids were self-pollinated, and phenotypes of the resulting progeny were evaluated. Phenotypic differences between the self-pollinated progeny of the reciprocal F₁ hybrids were significantly reduced compared with the reciprocal F₁ hybrids themselves for both traits. This suggested that there was only a negligible effect of cytoplasmic factors on the phenotypes observed (Fig. 4). Unlike endosperm tissue, the tissues used in this study (embryos at R5 stage and germinating embryo axes) were diploid, and, therefore, dosage of any given gene should be equal from both female and male parent. The genotype of the endosperm can have a significant effect on early embryo development (Chang and Neuffer, 1994), but, to our knowledge, it has not been shown to have an effect on embryos at later stages of development.

Imprinting Effect

We hypothesize that the phenotypic differences between the reciprocal F₁ hybrids observed in this study were most likely due to the imprinting phenomenon, and the differentially expressed genes identified were either directly or indirectly associated with such phenomenon. Imprinting was extensively studied in plants involving endosperm genes in maize (Kermicle and Alleman, 1990) and Arabidopsis (Luo et al., 2000; Russinova and de Vries, 2000). One of the best characterized imprinted loci of plants is the *R* locus that controls the anthocyanin pigmentation in maize aleurone. Using maize *B-A* translocation stocks with segmental duplications and deletions of chromosome 10L, Kermicle (1970, 1978) demonstrated that some of the *R* alleles condition mottled pigmentation in the aleurone after paternal transmission. Similarly, Lin (1982), also using maize *B-A* translocations, provided evidence for the im-

printing effect of endosperm factors (*Ef* genes) in normal endosperm growth. The *Ef* genes, some of which are also located on chromosome 10L, were shown to support normal growth of endosperm only when inherited from the male parent.

In this study, we observed various α -tubulin genes to be differentially expressed in embryo and germinating-embryo axis tissues between the reciprocal crosses in both CG (Table I) and DT (Table IV) traits. Lund et al. (1995b) reported differential demethylation of α -tubulin genes between embryo and endosperm tissues in maize where DNA demethylation correlated with the increased accumulation of *tub α 2* and *tub α 4* RNA. Furthermore, they also reported that the differential demethylation occurred in the endosperm tissue between reciprocal crosses of maize inbred lines (W64A and A69Y) and that the demethylation state of *tub α 3* and *tub α 4* was maternally transmitted.

The fertilization independent seed (*FIS*) genes (*MEA* or *FIS1*, *FIS2*, and *FIE* or *FIS3*) that repress Arabidopsis endosperm development in the absence of pollination are perhaps the most studied imprinted genes in plants at the molecular level (Grossniklaus et al., 1998; Luo et al., 2000). Earlier observations of imprinting (parent-specific transmission) of these genes have come from studying the reciprocal F_1 hybrids involving wild-type and mutant alleles of *FIS* genes (Ohad et al., 1996). Vielle-Calzada et al. (2000) recently reported that only maternal alleles were expressed during early embryogenesis (up to 64-cell stage embryo) and endosperm development in Arabidopsis. However, the authors also reported that the paternal alleles were expressed 3 to 4 d after fertilization. In this study, we demonstrate that embryos derived from seed of reciprocally cross-pollinated inbreds can display divergent phenotypes and differential patterns of gene expression, despite the fact that they are genetically identical.

mRNA Profiling

We used GeneCalling technology of CuraGen Corporation (New Haven, CT) to profile differences of mRNA expression between the reciprocal F_1 hybrids that displayed significant phenotypic divergence in their ability to tolerate high-temperature desiccation or germinate under cold conditions. Shimkets et al. (1999) showed that the GeneCalling profiling procedure could yield at least one detectable cDNA fragment from more than 90% of expressed genes with an average of 2.3 fragments per gene when 72 restriction enzyme pairs were used. In our study, we used 89 and 88 enzyme pairs for CG and DT traits, respectively, yielding >32,000 cDNA fragments in each case. Only a subset (< 2%) of these showed ≥ 1.5 -fold change in their expression between the reciprocal F_1 hybrids. Comparison of reciprocal F_1 s, which are

genetically identical (also, we sampled diploid tissue), helped eliminate the effect of DNA polymorphism that can significantly increase false positives (the number of cDNA fragments not relevant in influencing the trait) in the GeneCalling process.

A subset of the differentially expressed cDNA fragments was selected for further analyses via competitive PCR or cloning and sequencing. Twenty-four genes were positively confirmed from a total of 336 cDNA fragments that were differentially expressed (≥ 1.5 -fold change) between the high- and low-reciprocal F_1 s for CG (Table I). Similarly, the identification of 64 genes was positively confirmed out of 656 differentially expressed cDNA fragments between desiccation-tolerant and -sensitive reciprocal F_1 hybrids (Table II). The maximum expression fold change observed for the CG trait was 14.8 (Table I) and that in DT was 8.0 (Table II). BLAST analyses revealed that all the genes were in public databases, either in GenBank composite/expressed sequence tag (EST) or in SwissProt/TrEMBL, and belong to wide functional categories (Tables I and II).

Protein Profiling

Systematic survey of subcellular or whole-cell proteins by either N-terminal Edman sequencing or tandem mass spectrometry of individual spots resolved on a two-dimensional gel followed by database searching is fast becoming a common method of choice in developing maps of proteomes in various organisms, including plants (Peltier et al., 2000). To our knowledge, a survey of differentially expressed proteins that are attributable to a specific trait (phenotype) has never been reported in plants. In this study, we compared protein profiles of reciprocal F_1 hybrids that showed differential phenotypes for CG and DT traits.

Protein expression map (PEM) analysis with Oxford GlycoSciences' (Oxfordshire, UK) technology involved high-resolution quantitative detection (femtomol levels) of polypeptides followed by identification of proteins by database query using MS/MS spectral data (Page et al., 1999). In addition, PEM analysis can also provide insight into posttranslational modifications, proteolytic derivation, or isoforms of the same protein. For example, enolase 1 was annotated for two different protein features, numbers 2,518 and 2,519 (Table III; Fig. 7A), with a mass difference of 680 d and a pI difference of 0.1. These values suggest that the former protein is a proteolytic derivative of the latter or that they are isoforms of same enzyme, perhaps localized in different subcellular compartments. In maize, enolase 1 (*eno1*) is induced under anaerobic stress but *eno2* is constitutive. Lal et al. (1998) speculated that the regulation of these genes involves post-translational modification. MS/MS of a few of the protein features in this study were matched to two different proteins

each. Such annotations include protein feature numbers 4,413, 2,869, and 1,888 in Table III and 3,544 and 2,304 in Table IV. Some of these have common sequence motifs suggesting that they have functional similarity or that they belong to the same regulatory pathway.

Differentially Expressed Genes

The differentially expressed genes revealed by mRNA profiling and by protein profiling were different in both CG and DT traits. Nonetheless, several genes with similar functions and biochemical pathways were represented in both types of expression profiling. Examples include glycolytic/gluconeogenic pathway enzymes, members of cell wall and polyamine metabolism, ABA/stress-induced proteins, components of the cytoskeleton, chaperonins, etc. (Tables I–IV). Although we cannot know with certainty, perhaps the same genes were not observed by both profiling methods within each trait because only a subset of the total population of differentially expressed cDNA fragments and proteins were identified. A more comprehensive analysis of expressed proteins would require cellular fractionation and enrichment methods to identify low-abundance proteins.

Some of the genes observed in this study were previously shown to be associated with biotic and abiotic stress response in maize during seed development and germination. These include ABA/stress-inducible proteins, heat shock proteins, late embryogenesis abundant (LEA) proteins, and proteases (Yacoub and Filion, 1986; Prasad, 1996; Nieto-Sotelo et al., 1999; White et al., 2000). Upon comparing the accession numbers of drought and cold-induced genes in *Arabidopsis* reported by Seki et al. (2001) with the differentially expressed genes in this study (Tables I–IV), we found none of the genes to be in common. A closer examination by the gene description revealed that the expression of several genes, including LEA proteins, enolase, Cys protease, glyoxalase I, nodulin, heat shock protein dnaJ, and β -glucosidase were changed in both studies. Expression of these genes was modulated in the same direction in both the studies, except one of the LEA proteins, enolase, and glyoxalase I (Seki et al., 2001; Tables I–IV). This could be explained by differential embryo morphology, temporal and developmental differences, mono- and dicotyledonous nature, differences in experimental conditions, and differential responses of various isoforms of these genes to stress.

Protein profiling of reciprocal F_1 s for the CG trait showed modulation in expression of genes involved in protein degradation such as proteases and proteins associated with trafficking of ubiquitinated proteins to proteosomes (Fu et al., 1999; Tables I and III). These proteins may play a role in mobilization of proteins from the scutellum to the growing meris-

tems of the primary root and shoot during germination. Proteosome (26S and 20S) component proteins and proteases were also differentially expressed for the DT trait (Table II) and, in an analogous way, may be important in preconditioning the embryo so as to render it more tolerant of high-temperature stress during the final phase of maturation.

Protein expression profiling of reciprocal F_1 hybrids divergent for DT showed 30 different globulin polypeptides. That is more than double the number of previously reported globulin fractions (Cross and Adams, 1983a, 1983b). Globulins are encoded by *Glb1* and *Glb2* genes, which are present in only one or two copies each in the maize genome (Belanger and Kriz, 1991; Wallace and Kriz, 1991). However, the details of post-translational processing and assembly of such a large number of polypeptides into protein bodies remain unclear. It was interesting to note that the majority of globulin 1 fractions were down-regulated, whereas globulin 2 fractions were up-regulated in the desiccation-tolerant F_1 hybrid. Sequence alignment of *Glb1* and *Glb2* revealed no significant similarity. Further examination is required to understand the effect of globulins on DT in maize.

Some of the differentially expressed genes observed in this study were implicated in more than one function, including structural, enzymatic, and regulatory with various subcellular localizations. True functions of many of the genes are currently being studied. It is important to understand that both of these traits are complex in that the allelic condition of many genes at different loci, rather than a single or a few genes, may determine the phenotype. It is not surprising that these traits were associated with a large number of genes encompassing a broad range of biochemical pathways. This is, in fact, borne out by the mere number of genes we measured as differentially expressed in this tightly controlled comparison. It is reasonable to assume that pleiotropic effects of a subset of the differentially expressed genes caused altered expression of some of the other genes identified in this study. It is expected that among the genes identified as “differential” there lie those that are intimately involved in controlling cellular processes that lead to the manifestation of differences in CG and DT phenotypes.

MATERIALS AND METHODS

Germplasm Screening and Reciprocal F_1 Hybrid Production

Recombinant inbred populations were generated from a cross between B73 and MO17. The inbred lines were derived by self-pollinating the original F_1 plants (syn0) or sib-mating the original F_1 plants for two (syn2) or four (syn4) cycles, followed by self-pollinating each line of the F_2 progeny for at least 11 generations. These lines were screened for their ability to tolerate high desiccation temperature when harvested at high seed-moisture content (for DT) and to germinate under cold conditions (for CG and DT). The lab-based CG test was performed according to Byrum and Copeland (1995) with minor modifications. Seeds were germinated in rolls of wet

germination paper and incubated at 10°C for 7 d followed by 3 d at 27°C in the dark. Seedlings that had ≥ 2.5 cm shoot and ≥ 2.5 cm primary root lengths were considered germinated. In some cases, the primary root and shoot lengths were also recorded as a measure of seedling vigor (data not shown).

For the DT trait, 100 RILs that showed high germination under cold test conditions when harvested at low seed moisture (approximately 25%, R6 stage or physiological maturity) and dried at low (35°C/normal) temperature were screened. The screening was done by harvesting self-pollinated ears at low ($\leq 25\%$) or high ($\geq 40\%$, R5, or dent stage) seed-moisture content and drying at low (35°C, relative humidity [RH] 51%) or high (43°C, RH 28%) temperatures followed by performing the CG test. For the CG trait, self-pollinated ears harvested after physiological maturity from 780 RILs were screened using the CG test. Five contrasting RILs for each trait, DT (tolerant: M0002-syn0, M0021-syn4, M0212-syn4, M0241-syn0, M0363-syn4; and sensitive: M0079-syn0, M0213-syn0, M0247-syn4, M0337-syn4, M0367-syn2) and CG (high: M0029-syn2, M0062-syn0, M0081-syn0, M00324-syn0, M00367-syn2; and low: M0023-syn0, M0028-syn0, M00106-syn4, M0113-syn2, M0342-syn0), were selected for crossing. The divergent RILs were self-pollinated and cross-pollinated reciprocally in a diallel experimental design. Seeds from the selected reciprocally crossed ears (F_1 s) were grown and self-pollinated in the following season to test for cytoplasmic inheritance of the traits.

Phenotypic Analysis and Sample Collection

Self-pollinated and reciprocally cross-pollinated ears for the DT study were harvested at high (R5 stage) and low (R6 stage) seed moisture. After drying treatments at low (35°C, RH 51%) and high (43°C, RH 28%) temperatures, CG was evaluated on three individual ears for each treatment as described earlier. Similarly, five mature individual self- and reciprocally cross-pollinated ears were tested for the CG trait.

Specific combinations of RILs were identified based on the phenotypic analyses where the reciprocal F_1 hybrids displayed significant differences. In case of the DT trait, a few rows of kernels were removed from each ear before drying treatment to estimate moisture content and to dissect embryos (only from seeds harvested at 40% moisture content) for RNA and protein extraction. In case of the CG trait, embryo axes tissue of seed germinated for 11 d at 12.5°C in the dark were used to extract RNA and protein.

GeneCalling Analysis of mRNA from Reciprocal F_1 Hybrids

The GeneCalling procedure involves a comprehensive quantitative analysis of differentially expressed transcripts. Isolated mRNA is converted to double-stranded cDNA, which in turn is subjected to digestion with specific pairs of restriction enzymes (restriction fragments). Gene identification is accomplished by matching fragment size(s) using a computer algorithm to query against virtual digests of genes in a database (see Rothberg et al., 1999; Bruce et al., 2000). In brief, total RNA was isolated from 1 to 2 g of ground tissue using the PUREscript kit (Gentra Systems, Inc., Minneapolis). Isolation of poly(A⁺) RNA, cDNA preparations, restriction digestion, adapter ligation and PCR amplification, restriction fragment separation, comparison and identification of differentially expressed fragments, and gene identification and confirmation were performed according to Shimkets et al. (1999). The cDNA preparations of CG and DT samples were digested with 89 and 88 restriction enzyme pairs, respectively. Fragments from each digest were ligated to and PCR amplified with restriction enzyme-specific oligonucleotide adaptor/primer pairs. One of the primers was labeled with fluorescamine and the other with biotin. Labeled fragments were purified using streptavidin beads and were resolved by high-resolution capillary gel electrophoresis to generate traces showing peaks whose position and height represented M_r and abundance of cDNA fragment(s), respectively. Trace data were used for qualitative (M_r) and quantitative (abundance) comparisons between the samples from reciprocal F_1 hybrids.

The cDNA fragments that showed ≥ 1.5 -fold change between the reciprocal F_1 hybrids were identified from the trace database and were queried against publicly available maize (*Zea mays*) sequences and ESTs from the proprietary Pioneer/DuPont database for gene identification. The cDNA fragments that did not match any genes in the database were cloned from the original PCR reaction and sequenced using standard protocols. Genes

identified through the GeneCalling process or via cloning of fragments were confirmed by a competitive PCR method in which the original PCR reaction was re-amplified in the presence or absence of an excess quantity of unlabeled gene/sequence-specific primers (a few additional bases into the template). Gene identification was positively confirmed if competitive PCR caused the chromatographic peak of the particular cDNA species to completely disappear. On occasion, in cases where there was more than one cDNA species at a single point of the chromatogram (i.e. different cDNA fragments with the same M_r), then a partial reduction of peak height would occur, also confirming the gene identification.

All the cloned and sequenced cDNA fragments were subjected to BLASTX and BLASTN analyses by querying against public and proprietary databases (Altschul et al., 1990). Initial searches were performed against GenBank and SwissProt/TrEMBL, and genes with $\geq 93\%$ identity of the entire query fragment were recorded. Remaining sequences were searched in the EST databases, and the accession numbers were recorded.

Protein Expression Profiling

Proteomic analysis involved high-resolution separation on two-dimensional gels, gel image analysis, robotic excision, MS/MS analysis, and protein identification by querying virtual spectra of database proteins (Fig. 6). Page et al. (1999) described in detail various steps involved in this procedure. The same processes were followed in this experiment with minor modifications as described below.

Proteins from frozen ground tissue were extracted in a buffer containing 8 M urea, 2 M thiourea, 65 mM dithiothreitol, and 4% (w/v) CHAPS. After 4 h of extraction on a shaker at 4°C, the supernatants were subjected to two-dimensional gel electrophoresis consisting of isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension. Three gels were run for each sample. The gels were stained with fluorescent dye OGT MP17 that binds non-covalently to the SDS moiety attached to the protein, enabling features at sub-nanogram quantities to be visualized. High-resolution (200 μ m) scans of the gels were electronically stored in a database for image analyses. Each protein feature found across replicate gels was indexed (with pI and relative M_r) along with its average (across replicate gels) intensity, and recorded as percentage of the total feature intensity (percent volume). Such definition of all the features in a given sample across the replicate gels is referred to as PEM. Samples from three replicate ears from each reciprocal F_1 hybrid of a given trait were organized into a table by the protein feature index numbers using Melanie II software (Wilkins et al., 1996).

Differential protein expression between the reciprocal F_1 hybrids was analyzed using statistical methods. The raw percent volume data of each protein feature across replicate gels and samples of each F_1 genotype were subjected to analysis of variance. Features that showed significant percent volume difference ($P \leq 0.1$) between the reciprocal F_1 s and were clearly visible in the gel images of at least one genotype were used to calculate ratios indicating fold expression differences. Fold change in expression was derived by taking the ratio of mean percent volume of a given feature between the reciprocal F_1 samples. Proteins that showed ≥ 1.5 -fold change in expression were excised from the gel by a software-driven robotic cutter and delivered in a 96-well plate for proteolysis and MS analyses.

Excised proteins were subjected to trypsin digestion, and the mass of the oligopeptides were estimated by matrix-assisted laser-desorption/ionization time of flight mass spectrometer (ELITE, PerSeptive Biosystems, Framingham, MA). For MS/MS analyses, nano-electrospray ionization source (Z-spray) on a quadrupole time of flight instrument (Micromass, Manchester, UK) was used to generate fragmentation spectra. These uninterpreted MS/MS of the peptides were converted to centered spectra and used to query public (GenPept and SwissProt) and proprietary (Pioneer/DuPont and Oxford GlycoSciences) databases using a computer algorithm (SEQUEST program, Fennigan Corp.) based on the approach of Eng et al. (1994). The sequences of the oligopeptides were derived by matching the predicted ion series from a complete peptide sequence in the database consistent with the observed y-type fragmentation of peptide pools in the sample (Biemann, 1990).

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