Transcriptional and Metabolic Adjustments in ADP-Glucose Pyrophosphorylase-Deficient bt2 Maize Kernels¹[W]

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During the cloning of monogenic recessive mutations responsible for a defective kernel phenotype in a Mutator-induced Zea mays mutant collection, we isolated a new mutant allele in Brittle2 (Bt2), which codes for the small subunit of ADP-glucose pyrophosphorylase (AGPase), a key enzyme in starch synthesis. Reverse transcription-polymerase chain reaction experiments with gene-specific primers confirmed a predominant expression of Bt2 in endosperm, of Agpslz in embryo, and of Agpslz in leaf, but also revealed considerable additional expression in various tissues for all three genes. Bt2a, an alternative transcript coding for a plastidial isoform, was expressed in almost all tissues tested with a pattern very similar to that of Agpslz. The phenotypic analysis showed that, at 30 d after pollination (DAP), mutant kernels were plumper than wild-type kernels, that the onset of kernel collapse took place between 31 and 35 DAP, and that the number of starch grains was greatly reduced in the mutant endosperm but not the mutant embryo. A comparative transcriptome analysis of wild-type and bt2-H2328 kernels at middevelopment (35 DAP) with the 18K GeneChip Maize Genome Array led to the conclusion that the lack of Bt2-encoded AGPase triggers large-scale changes on the transcriptional level that concern mainly genes involved in carbohydrate or amino acid metabolism pathways. Principal component analysis of ¹H nuclear magnetic resonance metabolic profiles confirmed the impact of the bt2-H2328 mutation on these pathways and revealed that the bt2-H2328 mutation did not only affect the endosperm, but also the embryo at the metabolic level. These data suggest that, in the bt2-H2328 endosperms, regulatory networks are activated that redirect excess carbon into alternative biosynthetic pathways (amino acid synthesis) or into other tissues (embryo).

The maize (Zea mays) kernel represents one of the most important sources of human nutrition either directly, in the form of tortillas, chips, flakes, vegetable oil, or sweet corn, or indirectly, as energy-rich fodder for farm animals. All three parts of the kernel contribute to its nutritional value. The endosperm contains mainly starch and protein, the embryo is rich in fatty acids, and the surrounding pericarp provides fiber. These reserve substances are accumulated during the filling or maturation phase, which is preceded by the early or developmental phase and followed by the dehydration or desiccation phase. The knowledge of the molecular mechanisms governing the development, filling, and maturation of the maize kernel is in sharp contrast to its agronomic and economic importance. Despite considerable progress in recent years, the number of genes with a clearly identified function in kernel development remains extremely limited and is a long way short of the 1,000 genes estimated to be involved in seed development in Arabidopsis (Arabidopsis thaliana; McElver et al., 2001).

Genetic studies of the maize kernel started at the beginning of the last century. Together with Drosophila, maize was the favorite model of geneticists because the several hundred kernels on a single segregating ear allowed easy scoring of traits concerning the color, size, or shape of the kernel. Not surprisingly, some of these mutants turned out to lack genes involved in the synthesis of the reserve substances that constitute the nutritional interest of the maize kernel. Kernel mutants were originally obtained spontaneously (Mains, 1949), later on by ethyl methanesulfonate mutagenesis.
(Neuffer and Sheridan, 1980), and, more recently, by insertionional mutagenesis (Clark and Sheridan, 1991; Scanlon et al., 1994). Mutant characterization was first limited to cytological descriptions, later on to biochemical quantifications of metabolites or enzyme activities, and only more recently to the cloning of the underlying genes. For example, the shrunken2 (sh2) mutant was isolated in 1949 (Mains, 1949). The lack of starch in the mature kernel was demonstrated in 1953 (Laughnan, 1953) and the absence of ADP-Glc pyrophosphorylase (AGPase) activity was demonstrated in 1966 (Tsai and Nelson, 1966), whereas the isolation of the mutated gene had to wait until 1990 (Bhave et al., 1990).

In addition to a dozen mutants involved in starch synthesis, several developmental mutations have been cloned. Mutations in the two plastid ribosomal proteins L35 (Magnar et al., 2004) and S9 (Ma and Dooner, 2004) lead to lethality of the early embryo, but do not influence endosperm development. The characterization of empty pericarp (emp) mutants showed that loss of the negative regulator of the heat shock response Emp2 (Fu et al., 2002) or the mitochondrial-targeted pentatricopeptide repeat protein Emp4 (Gutierrez-Marcos et al., 2007) affects both embryo and endosperm development. Lesions in the Cys proteinase Dek1 (Lid et al., 2002) cause a defective kernel phenotype similar to the one observed after loss of the transmembrane protein Lachrima (Stiefel et al., 1999). Finally, the analysis of etched (et) mutants indicated that the zinc ribbon protein Etched1 (da Costa e Silva et al., 2004) is needed to avoid fissures and cracks in the maize kernel. Many more genes transcribed in the maize kernel have been characterized in great detail. Even though in many cases rather precise functions can be attributed to these genes by sequence homology to proteins of known function, these putative functions need to be demonstrated by the analysis of corresponding mutants or transgenic knockouts.

Among all the developmental and biosynthetic pathways of the maize kernel, the one leading to starch is certainly the best characterized. Most genes involved in the major steps of starch synthesis have been identified by mutant analysis. Along the pathway leading from Suc via activated hexoses to the starch polymer we find, in order, Miniature1 (Mnt1) coding for the cell wall invertase IncW2, Shrunken1 (Sh1) and Sucrose synthase1 (Sus1) coding for Suc synthase, Sh2 and Brittle2 (Bt2) coding for AGPase, Bt1 coding for an ADP-Glc transporter, Waxy1 (Wx1) coding for the granule-bound starch synthase GBSSI, Sugary2 (Su2) and Dull1 (Du1) coding, respectively, for the soluble starch synthases SSIIa and SSIIIa, Amylose extender1 (Ael1) coding for branching enzyme BElII, and St1 coding for the debranching enzyme ISA1 (Hannah, 2005, and refs. therein).

The rate-limiting step in starch synthesis is the synthesis of ADP-Glc from Glc-1-P and ATP by AGPase (EC 2.7.7.27; Russell et al., 1993). AGPase is a heterotetramer composed of two large subunits encoded by Sh2 and two small subunits encoded by Bt2. Homozygous sh2 or bt2 kernels collapse (shrink) during the maturation process, becoming angular (brittle) at maturity when they contain very little starch and strongly increased amounts of Suc (Laughnan, 1953; Cameron and Teas, 1954). Whereas the presence of two different subunits is typical for higher plants, both photosynthetic and nonphotosynthetic bacteria have only a single AGPase gene. All genes have evolved from a common ancestor and substantial sequence similarity remains between Sh2 and Bt2, although it is smaller than the similarity between Bt2 and genes encoding small subunits in other species (Smith-White and Preiss, 1992). Both maize genes individually have small but detectable catalytic activity in Escherichia coli (Burger et al., 2003), questioning earlier reports on potato (Solanum tuberosum) AGPase genes that attributed catalytic activity only to the small subunit (Ballesta et al., 1995).

The presence of residual AGPase activity in sh2 (12%) and bt2 (17%) kernels was the first indication for additional AGPase genes in the maize genome (Dickinson and Preiss, 1969) and led ultimately to the isolation of two additional genes from an embryo cDNA library (Giroux and Hannah, 1994). Agp1 (large subunit) and Agp2 (small subunit) show predominant expression in the embryo in northern blots, contrary to Sh2 and Bt2, which are specifically expressed in the endosperm (Giroux and Hannah, 1994). Finally, a third small subunit gene, L2, was identified in a leaf cDNA library (Prioul et al., 1994). The genes Agp2 and L2 were renamed Agps1zm and Agps2zm, respectively, when the intron/exon structure of Agps1zm was determined (Hannah et al., 2001). Although the three Agps genes clearly have a common ancestor, their physiological roles have diverged after gene duplication. Whereas Agps1zm is responsible for the transient accumulation of starch during the daytime in the source organ leaf, Agps2zm and Bt2 take care of the permanent storage of starch in the sink organs embryo and endosperm.

Another point of divergence between the three Agps genes is their subcellular localization. Although it has been a matter of long-standing debate, there is now growing consensus that the Bt2 protein is located in the cytoplasm rather than the plastids of endosperm cells. The gene lacks transit peptide sequences, the protein does not seem to be processed (Giroux and Hannah, 1994), 95% of the AGPase activity of endosperm cells is found in the cytosolic fraction (Denyer et al., 1996), and Bt2-GFP fusions locate to the cytoplasm of transgenic tobacco (Nicotiana tabacum) plants (Choi et al., 2001). The cytoplasmic localization of Bt2 is shared by its counterparts in barley (Hordeum vulgare; Thorbjornsen et al., 1996a), wheat (Triticum aestivum; Burton et al., 2002) and rice (Oryza sativa; Ohdan et al., 2005), but differs fundamentally from the plastid localization of dicot AGPS proteins and monocot nonendosperm AGPS proteins (Beckles et al., 2001). The authors speculate that a cytosolic AGPase has the advantage of committing carbon to starch synthesis rather than other metabolic pathways in the presence of high Suc levels.
The objective of our research program is the identification of genes causing an emphy phenotype of the maize kernel and the phenotypic characterization of the corresponding mutants. We report here the isolation of a novel insertional allele of \( btt \), as well as a detailed molecular and phenotypic characterization of the \( btt-H2328 \) mutant, including gene-specific reverse transcription (RT)-PCR, transcriptome and metabolome analyses never performed before on \( btt \) mutants.

**RESULTS**

**Isolation and Molecular Characterization of a Mutator Insertion in \( Btt \)**

During a forward genetics approach aimed at the systematic cloning of monogenic recessive mutations responsible for kernel phenotypes in the Mutator (Mu)-induced Biogemma mutant collection (Martin et al., 2006), the flanking sequences H6P (EU137670) and H6P' (EU137671) showed cosegregation with the kernel phenotype of mutant H2328. Genetic mapping of H6P on chromosome 4 in BIN 4.04/4.05 between markers UMC49d and PHI026 incited us to test whether mutant H2328 was able to complement the \( btt \) mutation, a well-known kernel mutation with a very similar map position. Absence of complementation led us to the conclusion that there was close linkage between H6P, H6P', and \( Btt \) and that a mutation in \( Btt \) rather than H6P' was responsible for the phenotype of H2328.

In the search for a molecular lesion in the \( Btt \) gene of H2328, gene-specific primers were used either in pairs to yield four PCR fragments that covered nearly all of the \( Btt \) gene or in combination with a \( Mu \) primer in a more specific search for \( Mu \) insertions (see "Materials and Methods"). PCR on genomic DNA of wild-type, heterozygous, and homozygous mutant plants revealed a \( Mu \) insertion in one of the four fragments. Cloning and sequencing of the flanking sequences of the insertion showed that the insertion was located in exon 6 of the \( Btt \) gene generating a 9-bp duplication of bases TGATGTGAC (position 4,422–4,431 in accession no. AF334959). Perfect cosegregation between this insertion and the kernel phenotype of mutant H2328 was found on a segregating population of 66 individuals. From here on, this novel \( btt \) allele will be referred to as \( btt-H2328 \).

**Phenotypic Analysis**

Mature \( btt \) kernels contain essentially a seemingly normal embryo, whereas the surrounding endosperm is completely collapsed. Numerous phenotypic analyses have been performed on \( btt \) mutants, but most of them focused on mature kernels or biochemical features. For cytological and molecular analysis of \( btt \) kernels throughout development, it seemed important to have an easy means to distinguish wild-type from mutant kernels. Following up on earlier observations on \( sh2 \) kernels, we marked the two size classes visible on immature 30-DAP kernels with black dots (bigger, plumper) or red dots (smaller, slight depression; Fig. 1A). Observation at maturity showed that all kernels with black dots were mutant, whereas kernels with red dots were wild type (Fig. 1B). Similar observations were made on segregating ears of mutants H182, H816, \( btt \), and, to a lesser extent, \( sh2 \) (data not shown).

To determine more precisely the exact timing of the onset of the kernel collapse, homozygous mutant \( btt-H2328 \) plants were grown in the greenhouse, self-pollinated, and observed throughout kernel development. As early as 31 DAP, a small depression was seen on some kernels, mainly toward the tip of the ear (Fig. 1C). With time, the depressions became deeper and new depressions appeared on additional kernels (Fig. 1D). By 40 DAP, almost all kernels showed at least a small depression (Fig. 1E) and, by 45 DAP, all kernels were concerned. These data demonstrated a certain heterogeneity of the phenotype, which possibly reflected slight differences between the nutritional or developmental status of individual kernels.

![Figure 1. Macroscopic phenotype of \( btt-H2328 \). A and B, On a segregating ear of \( btt-H2328 \), wild-type kernels were marked with a black dot and mutant kernels with a red dot. The ear was photographed at 30 (A) and 60 DAP (B). Mutant kernels were plumper than wild-type kernels at 30 DAP and collapsed at 60 DAP. C to F, The same ear of a self-pollinated \( btt-H2328 \) homozygous mutant plant was photographed at 31 (C), 35 (D), 40 (E), and 45 DAP (F). Black arrows, Mutant kernels already collapsed at 31 DAP; red arrows, mutant kernels still plump at 40 DAP.](image-url)
Cytological observations of bt2-H2328 mutant kernels before kernel collapse confirmed that important differences existed between mutant and wild-type kernels at 30 DAP (Fig. 2, A and B). There was a strong reduction of starch grain number and size in endosperm cells of bt2-H2328 mutant kernels (Fig. 2, B and E). Only the endosperm seemed to be affected and not the embryo (Fig. 2, C and F). In fact, the overall aspect of mutant endosperm cells was reminiscent to that of mutant and wild-type embryo cells. At 30 DAP, the differences in the endosperm were strongest in the apical (Fig. 2, I and M) and central (Fig. 2, J and N) parts and less pronounced in the basal (Fig. 2, G and K) and lateral parts (Fig. 2, H and L) of the endosperm.

Structure of Agps Genes

There has been a long-standing debate concerning the subcellular localization of AGPase in maize endosperm. Both cytoplasmic (Denyer et al., 1996) and plastid localization (Echeverria et al., 1988) have been reported. In addition, alternative splicing of a single gene has been known to be at the origin of cytoplasmic and plastid isoforms in barley (Thorbjornsen et al., 1996b), wheat (Burton et al., 2002), and rice (Ohdan et al., 2005). Interestingly, our in-depth analysis of the corresponding genomic sequences showed that the first intron of Bt2 contained a 255-bp sequence with 89% homology to the end of exon 1 of Agpslzm (Fig. 3). Using a start codon at the beginning and a splice site at the end of the conserved sequence, we derived a theoretical amino acid sequence of BT2 that carried an alternative N terminus (Fig. 3, BT2b). An interrogation of the TargetP Web site (http://www.cbs.dtu.dk/services/TargetP) predicted BT2b to be targeted to the plastid with a score of 0.971, just like AGPSLZM (score 0.946) or APSEMZM (score 0.862), whereas BT2a was predicted to remain in the cytosol (score 0.184 for plastid, score 0.770 for other). Very similar findings have been published recently by other authors (Rosti and Denyer, 2007).

Because the ongoing sequencing effort of the maize genome provided a genomic sequence for Agpsemzm, we extended an earlier study concerning the gene structure of maize Agps genes (Hannah et al., 2001). On that occasion, the existing Bt2 sequence was extended at its 5′-end and the 5′- and 3′-untranslated regions (UTRs) redefined by comparison to EST sequences (see “Materials and Methods”). The sequence comparisons showed that Agpsemzm had a conserved structure composed of 10 exons and nine introns. Sequence similarity to Bt2 and Agpslzm was moderate in exon 1 (43% and 68% in the coding sequences of exon 1) and the noncoding exon 10 (52% and 54%), and high in all

Figure 2. Cytological observations of bt2-H2328 kernels at 30 DAP. Wild-type and homozygous mutant kernels from a segregating ear were collected at 30 DAP. Paraffin sections were stained by the periodic acid-Schiff procedure coloring starch grains in pink. Global views (A and D), high magnifications (B, C, E, and F), and low magnifications (G–N) of embryo (emb) and endosperm tissue (en) are presented.
other exons (81%–91%) with highest similarities in exons 5 and 6. Not a single intron was conserved in length between any of the three Agps genes, with sequence similarities ranging from 44% to 59%.

Anchoring of the Agps genes on the physical map of maize allowed the establishment of synteny between the regions encompassing Bt2 (chromosome 4, BIN 4.05, ctg 165) or Agpslzm (chromosome 1, BIN 1.07, ctg 44) and a single region in the rice genome (chromosome 8, Os08g0345800), on one hand, and between the region containing Agpsemzm (chromosome 2, BIN 2.06, ctg 92) and a second region in the rice genome (chromosome 9, Os09g0298200), on the other hand. The two rice regions corresponded to the only two Agps genes present in the rice genome, which code for OsAgps1 (Os09g0298200) targeted to the plastid and OsAgps2 (Os08g0345800) with dual plastidial and cytoplasmic targeting (Lee et al., 2007). These data confirmed on the physical map recent observations made on the genetic map (Rosti and Denyer, 2007), and supported the conclusion of the authors that Bt2 and Agpslzm arose from gene duplication after the separation of maize and rice.

Expression of Agps Genes

We designed gene-specific primers to gain further insight into the expression level of the three Agps genes in major maize tissues, in different kernel compartments, and at various stages of kernel development. Control experiments demonstrated the specificity of the primer targets to the plastid and OsAgps2 (Os08g0345800) with dual plastidial and cytoplasmic targeting (Lee et al., 2007). These data confirmed on the physical map recent observations made on the genetic map (Rosti and Denyer, 2007), and supported the conclusion of the authors that Bt2 and Agpslzm arose from gene duplication after the separation of maize and rice.

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pairs by cloning and sequencing of PCR products and the absence of genomic contamination by the use of unrelated intron-spanning control primers (see “Materials and Methods”). In wild-type plants, the three Apgs genes were not tissue specific, although they showed preferences in terms of their spatial and temporal expression pattern (Fig. 4). As expected, Bt2 was strongly expressed in kernels, but moderate expression was also found in ears and tassels and weak expression in leaves. Separate analysis of Bt2a (cytoplasmic) and Bt2b (plastidial) allowed attribution of most of the kernel transcript to Bt2a and most of the transcript seen in other tissues to Bt2b. For Agpslzm, we confirmed much stronger expression in leaves than in kernels and demonstrated additional expression of comparable strength in ears and tassels. Agpsemzm indeed showed moderate expression in the kernel, but even higher expression was discovered in ears. It was weakly expressed in leaves, stems, and even roots (Fig. 4A).

In the kernel, Bt2 was predominantly expressed in the developing endosperm, where almost exclusive expression of Bt2a, rather than Bt2b, was observed. Lower Bt2 expression was also found in the embryo, mainly due to Bt2b. In contrast to the overall Bt2 pattern, Agpslzm was expressed only in the embryo and not in the endosperm, whereas Agpsemzm was clearly expressed both in the embryo and the endosperm, especially at early stages. All three genes showed maximal expression in their respective domains at 12 DAP, which steadily declined during the filling phase (35 DAP) to disappear during desiccation (70 DAP; Fig. 4B).

Transcript levels in the bt2-H2328 mutant were assessed both in leaves of wild-type and mutant sister plants and in 35-DAP kernels of segregating ears (Fig. 4C). The mutant was not a complete knockout because Bt2 was expressed in mutant kernels albeit at lower levels than in wild-type controls. The reduction concerned both splice forms Bt2a and Bt2b. Unexpectedly, the expression of Agpslzm and Agpsemzm was also affected in the bt2-H2328 mutant (Fig. 4C). Quantification by quantitative RT-PCR indicated down-regulation by a factor of 14 (Bt2), a factor of 3 (Agpslzm), or a factor of 2 (Agpsemzm) in bt2-H2328 kernels (data not shown).

Taken together, these results showed that Bt2a, Agpslzm, and Agpsemzm were preferentially but not specifically expressed in endosperm, leaf, and embryo, respectively. Bt2b was expressed in almost all tissues tested and had a very similar expression pattern as Agpslzm. The results suggested also that the residual activity of AGPase in mutant kernels of many bt2 alleles could be readily explained by the expression of Agpslzm, Agpsemzm, and/or residual Bt2 expression.

Transcriptome Analysis

Despite a wealth of knowledge on the enzymology and allosteric regulation of maize AGPase in wild-type and bt2 kernels, only very limited information was available on the consequences of AGPase dysfunction on gene expression (Giroux et al., 1994). To investigate in a nonbiased fashion whether the block of starch synthesis and the subsequent accumulation of Suc...
caused adjustments of metabolic pathways on the transcriptional level, a microarray experiment using the 18K GeneChip Maize Genome Array (Affymetrix) was carried out. Whole kernels (pericarp + endosperm + embryo) from self-pollinated ears of heterozygous plants (+/bt2-H2328) were collected at 35 DAP and visually divided into pools of wild-type and mutant kernels. Two different ears were used to obtain a biological duplicate.

The statistical analysis resulted in a large number of differentially expressed genes. A total of 2,345 of the 17,734 probe sets present on the array were declared differentially expressed (Bonferroni P value <0.05). These genes showed either increased or decreased expression with an absolute value of log2 ratio >1.08. We then asked the question whether certain Gene Ontology (GO) terms were overrepresented among differentially expressed probe sets in comparison to all probe sets. Not surprisingly, polysaccharide metabolic processes (GO:0005976) and related terms showed a statistically significant (P value <0.01) increase among differentially expressed probe sets (Table I). Interestingly, amino acid metabolic processes (GO:0006520) and related terms were even more frequent in the list of differentially expressed probe sets (Table I). On the other hand, there was no significant difference in the case of lipid metabolic processes (GO:0006629), protein metabolic processes (GO:0019538), or transcription (GO:0006350). A further dissection of amino acid metabolic processes revealed that the changes in the transcriptome concerned the synthesis, rather than the catabolism, of amino acids and that most amino acid families were concerned, even though at various degrees (Table I).

An in-depth analysis of 51 genes involved in carbohydrate metabolism revealed differential expression for 24 of them (Table II). Of the 180 probe sets present on the array, 36 showed differential expression, 111 did not change expression levels, and for 33 no conclusion could be reached because the expression levels were too close to background. Down-regulated genes coded mainly for enzymes or transporters in starch biosynthesis (8/12; Fig. 5, pink), whereas up-regulated genes were more frequently involved in glycolysis, tricarboxylic acid (TCA) cycle, or pentose phosphate pathway (PPP; 8/9; Fig. 5, green). In three cases, both up- and down-regulated probe sets were found for a single enzymatic step (Fig. 5, yellow). The probe sets with diverging expression patterns corresponded clearly to distinct genes with redundant function, possibly reflecting isoforms with preferential activity in certain tissues or subcellular compartments.

For the analysis of amino acid metabolism, the starting point was the GO term amino acid metabolic processes (GO:0006520). Of the 61 probe sets annotated with this term, 34 were differentially expressed, 24 were unchanged, and three undistinguishable from background. Consistent up-regulation was found for genes related to the biosynthesis of aromatic amino acids (Trp, Tyr, and Phe), whereas the picture was more complex with some up- and some down-regulated steps in the biosynthetic pathways of other amino acids (Fig. 5). Again, there were two examples of up- and down-regulated genes coding for the same enzymatic function soliciting the same explanations as with carbohydrate metabolism.

To see whether these changes in amino acid metabolism had consequences on the transcriptional regulation of storage proteins, we checked the expression of 17 Zein genes as well as of a Legumin and a Globulin gene as classified earlier (Woo et al., 2001). Among the 51 corresponding sense probe sets on the array, not a single one was up-regulated in bt2-H2328 kernels, whereas 23 were down-regulated, 23 showed no change, and five were indistinguishable from background (data not shown).

Taken together, these data indicate that the lack of Bt2-encoded AGPase triggers large-scale changes on the transcriptional level that concerns mainly genes involved in carbohydrate or amino acid metabolic pathways.

**Metabolome Analysis**

To provide further insight into the impact of the bt2-H2328 mutation on the metabolome of the developing maize kernel, a quantitative analysis by 1H NMR spectroscopy was carried out on wild-type and mutant kernels of three segregating ears at 35 DAP. For each ear, a pool of 10 wild-type kernels and a pool of 10 mutant kernels were constituted to have a biological triplicate. For each sample, two extractions were made in parallel to obtain a technical duplicate. In parallel, 15 additional wild-type kernels and 15 additional mutant kernels of each ear were micropo-dissected into embryo, on one hand, and endosperm (plus adhering pericarp), on the other, and pooled.

In the first instance, the NMR signatures (see Supplemental Figs. S1–S3) were analyzed by principal component analysis (PCA) of 0.04-ppm intervals of the entire spectra with the exclusion of the water and ethanol regions (see Supplemental Figs. S4 and S5). Comparing wild-type and bt2-H2328 endosperm, the first two PCA components explained 94% of total variability. PC1 with PC2 clearly separated the wild-type from the mutant samples. Examination of PC1 and PC2 loadings suggested that the difference between the wild-type and bt2-H2328 samples involved Suc, Fru, malate, and Ala. In the embryo, the first two PCA components explained 90% of total variability. PC1 with PC2 clearly separated the mutant from the wild-type samples. Examination of PC1 and PC2 loadings suggested that the difference between the wild-type and mutant samples involved lactic acid, Suc, citrate, lipids, and Pro. These data clearly demonstrated that the bt2-H2328 mutation did not only affect metabolite levels in the endosperm, but also in the embryo.

In parallel, 18 to 21 individual metabolites identified in the NMR signatures were quantified (see Supplemental Tables S1–S3). t tests allowed the pinpointing of
metabolites with statistically significant differences between wild-type and bt2-H2328 kernels, endosperms, and embryos (Tables III–V). Whereas increased levels of Suc and reducing sugars had been described repeatedly for bt2 kernels (Cameron and Teas, 1954), many of the other listed metabolites had never been measured before. Increases in citrate, malate, and fumarate levels indicated an influence of the bt2-H2328 mutation on the TCA cycle, whereas increases in Ala, Val, and Tyr levels revealed compensation in amino acid metabolism. Whereas these changes mainly concerned the endosperm, the embryo did not only share some of the changes (citrate, choline, Suc), but also showed its proper adjustments, in particular in lipid metabolism.
We present here the isolation as well as the molecular and phenotypic characterization of *bt2-H2328*, a new mutant allele of the *Bt2* gene coding for the small subunit of AGPase, a key enzyme in starch synthesis. The isolation of the mutation, which was the result of a systematic effort to identify *Mu* insertions causing a *mn* or an *emp* phenotype, was the occasion to revisit this gene and to complement earlier findings focusing in carbon and amino acid metabolism.

### Table II. Differentially expressed genes coding for enzymes involved in carbon and amino acid metabolism

Numbers in column 1 and colors in columns 1, 4, 5, and 6 correspond to the metabolic scheme in Figure 5. BGD, Background. *, No EC number.

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<th>EC No.</th>
<th>Enzyme Name</th>
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<th>Down in <em>bt2-H2328</em></th>
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<td>Isocitrate dehydrogenase</td>
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<td>Succinate dehydrogenase</td>
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<tr>
<td>B *</td>
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<td>Glc-6-P translocator</td>
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<td>EC 6.3.1.2</td>
<td>Gin synthetase</td>
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<td>EC 2.7.2.4</td>
<td>Asp kinase homo-Ser dehydrogenase</td>
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<td>EC 4.2.1.52</td>
<td>Dihydrolipicolinate synthase</td>
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<td>Dihydrolipicolinate reductase</td>
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<td>EC 2.1.1.13</td>
<td>Met synthase</td>
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</tbody>
</table>
Figure 5. Summary of transcriptional and metabolic changes in bt2-H2328 kernels. The scheme shows selected pathways of the carbohydrate and amino acid metabolism. Glycolysis, starch synthesis, TCA, and PPP are surrounded by circles; the different amino acid biosynthetic pathways are not. Solid arrows indicate a single enzymatic step, dashed arrows more than one enzymatic step. Enzymes are numbered according to Table II. Enzymes encoded by genes over- or underexpressed in bt2-H2328 kernels are highlighted in green and pink, respectively. Yellow highlighting indicates that at least one corresponding gene was overexpressed and at least one corresponding gene is underexpressed. Metabolites showing higher accumulation in bt2-H2328 than in wild-type kernels, endosperms, or embryos are highlighted in green.
mainly on the biochemistry and genetics of Bt2 (for review, see Hannah, 2005). Gene and allele-specific RT-PCR provided individual expression data for the two splice variants of Bt2 and the related genes Agpslzm and Agpsemzm, thus assessing on the transcriptional level the relative contribution of the three genes to AGPase activity in different tissues. Innovative transcriptome and metabolome studies revealed a strong influence of the bt2-H2328 mutation both on transcript and metabolite levels that concerned primarily carbohydrate and amino acid metabolism; in addition, the bt2-H2328 mutation did not affect exclusively the endosperm, but also influenced metabolite levels in the embryo. This study also highlights the difficulties of forward genetics with Mu-induced populations. A systematic transposon display approach on 40 kernel mutants yielded only one insertion cosegregating with the phenotype, which in the end turned out to be only tightly linked to, but not causal, for the phenotype. Whereas technical problems related to the high copy number and high somatic transposition rate of Mu certainly contributed to this very limited success, we also hypothesize that the active Mu stock used for the initial mutagenesis may have promoted the transposition of other elements not detected by our Mu-oriented transposon display, increased the frequency of single base mutations, or provoked epigenetic alterations.

Agps Expression in Maize Tissues

Our gene-specific RT-PCR expression data of the three Agps genes in major maize tissues and in kernel compartments throughout development enlarge and complement earlier studies based on enzymological or immunological differences of AGPase proteins in certain tissues or size differences of Agps transcripts in mutant kernels (Preiss et al., 1971; Giroux and Hannah, 1994). Whereas expression is strong in the tissues, where Bt2 (endosperm), Agpsemzm (embryo), and Agpslzm (leaf) were originally isolated, this expression is by no means specific. For example, we reveal substantial expression of all three genes in immature and mature ears and tassels. In the kernel, we document expression of Agpslzm in the embryo and of Agpsemzm in the endosperm. Taken together, these data indicate that Agps genes may play important roles in organs

<p>| Table III. Metabolite levels in wild-type and bt2-H2328 kernels at 35 DAP |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wild Type</th>
<th>Mutant</th>
<th>P Valuea</th>
<th>Ratio Mutant/Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc</td>
<td>50.02 ± 8.29</td>
<td>102.91 ± 19.01</td>
<td>9.56E-05</td>
<td>2.06</td>
</tr>
<tr>
<td>Ala</td>
<td>1.48 ± 0.12</td>
<td>2.33 ± 0.21</td>
<td>6.80E-06</td>
<td>1.57</td>
</tr>
<tr>
<td>Choline</td>
<td>0.50 ± 0.03</td>
<td>0.78 ± 0.11</td>
<td>1.09E-04</td>
<td>1.57</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.82 ± 0.19</td>
<td>5.73 ± 0.44</td>
<td>1.92E-06</td>
<td>1.50</td>
</tr>
<tr>
<td>Glc</td>
<td>6.55 ± 0.20</td>
<td>9.39 ± 0.55</td>
<td>3.03E-07</td>
<td>1.43</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.32 ± 0.16</td>
<td>1.86 ± 0.29</td>
<td>2.21E-03</td>
<td>1.41</td>
</tr>
<tr>
<td>S</td>
<td>2.44b</td>
<td>3.59 ± 0.10</td>
<td>4.87 ± 0.21</td>
<td>7.90E-08</td>
</tr>
<tr>
<td>Malate</td>
<td>9.48 ± 0.30</td>
<td>11.57 ± 0.39</td>
<td>1.06E-06</td>
<td>1.22</td>
</tr>
<tr>
<td>GABA</td>
<td>1.88 ± 0.07</td>
<td>2.26 ± 0.16</td>
<td>4.03E-04</td>
<td>1.20</td>
</tr>
<tr>
<td>Val</td>
<td>1.35 ± 0.04</td>
<td>1.55 ± 0.06</td>
<td>4.92E-05</td>
<td>1.15</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.91 ± 0.07</td>
<td>3.21 ± 0.11</td>
<td>2.05E-04</td>
<td>1.10</td>
</tr>
<tr>
<td>Fumarate</td>
<td>1.82 ± 0.01</td>
<td>1.99 ± 0.02</td>
<td>3.00E-09</td>
<td>1.09</td>
</tr>
</tbody>
</table>

a t test of biological triplicate and technical replicate; only metabolites with P value <0.01 are shown. b Unidentified lipid with peak at 4.9 ppm.

<p>| Table IV. Metabolite levels in wild-type and bt2-H2328 endosperms at 35 DAP |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Wild Type</th>
<th>Mutant</th>
<th>P Valuea</th>
<th>Ratio Mutant/Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc</td>
<td>46.26 ± 6.06</td>
<td>134.38 ± 46.06</td>
<td>7.78E-04</td>
<td>3.04</td>
</tr>
<tr>
<td>Citrate</td>
<td>4.73 ± 0.29</td>
<td>8.64 ± 1.37</td>
<td>4.52E-05</td>
<td>1.82</td>
</tr>
<tr>
<td>Choline</td>
<td>0.44 ± 0.02</td>
<td>0.63 ± 0.07</td>
<td>8.84E-05</td>
<td>1.42</td>
</tr>
<tr>
<td>S</td>
<td>2.44b</td>
<td>3.57 ± 0.09</td>
<td>5.06 ± 0.54</td>
<td>5.71E-05</td>
</tr>
<tr>
<td>Glc</td>
<td>6.54 ± 0.22</td>
<td>9.09 ± 0.27</td>
<td>1.00E-06</td>
<td>1.36</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.13 ± 0.05</td>
<td>1.54 ± 0.17</td>
<td>2.20E-04</td>
<td>1.36</td>
</tr>
<tr>
<td>Malate</td>
<td>9.32 ± 0.21</td>
<td>12.18 ± 0.82</td>
<td>8.96E-06</td>
<td>1.31</td>
</tr>
<tr>
<td>Ala</td>
<td>1.36 ± 0.10</td>
<td>1.67 ± 0.21</td>
<td>9.43E-03</td>
<td>1.23</td>
</tr>
<tr>
<td>Val</td>
<td>1.30 ± 0.02</td>
<td>1.44 ± 0.06</td>
<td>2.42E-04</td>
<td>1.12</td>
</tr>
<tr>
<td>Fumarate</td>
<td>1.84 ± 0.02</td>
<td>2.01 ± 0.02</td>
<td>3.00E-08</td>
<td>1.10</td>
</tr>
<tr>
<td>Asp</td>
<td>4.31 ± 0.06</td>
<td>4.47 ± 0.06</td>
<td>1.13E-03</td>
<td>1.04</td>
</tr>
</tbody>
</table>

a t test of biological triplicate and technical replicate; only metabolites with P value <0.01 are shown. b Unidentified compound with peak at 2.44 ppm.
other than the classical source (leaf) and sink organs (kernel) and that residual AGPase activity found in bt2 kernels can be attributed not only to \textit{Agpsemzm}, but also to \textit{Agpslzm}.

\textbf{Agps Gene Structure and Evolution}

Alternative splicing of the first intron of \textit{Agps} genes leading to either cytoplasmic or plastid-targeted isoforms of AGPase is well documented in barley (Thorbjornsen et al., 1996b), wheat (Burton et al., 2002), and rice (Lee et al., 2007). We confirmed and extended very recent findings of alternative splicing of \textit{Bt2} in maize (Rosti and Denyer, 2007) by splice variant-specific RT-PCR in all major maize tissues. The expression of \textit{Bt2a}, the variant coding for the cytoplasmic isoform, was limited to the kernel and, more precisely, to the developing endosperm up to the dehydration stage, substantiating earlier hypotheses that the cytoplasmic isoform, which is not found outside the grass tribe, is a particularity of grass endosperm, where it may have a functional role in partitioning large amounts of carbon into starch when Suc is abundant (Beckles et al., 2001). In contrast, \textit{Bt2b} was expressed at various levels in almost all tissues analyzed and its overall expression pattern was very similar to \textit{Agpslzm}. This is additional support for the model of Rosti and Denyer (2007), which claims that \textit{Bt2} and \textit{Agpslzm} arose by gene duplication from a common ancestor after the separation of maize from rice, wheat, and barley, that \textit{Agpslzm} subsequently lost the capacity to code for the cytoplasmic isoform and specialized in coding for the plastid isoform, whereas \textit{Bt2} maintained the capacity to code for both isoforms, but to some extent specialized in coding for the cytoplasmic one. A closer look at the upstream sequences of \textit{Agpslzm} and \textit{Bt2b} reveals substantial sequence similarity of 90% (not counting the numerous gaps) in the 200 bp immediately upstream of the putative ATGs, which drops to 48% in the 1,800 bp further upstream. Based on a clone from the maize full-length cDNA project (accession no. DR969810), the 5’-UTR of \textit{Agpslzm} is 130 bp. Consequently, conserved cis-elements are either located very close to the transcription initiation site in the highly conserved region or in small clusters of conserved nucleotides found in the region with lower overall homology. In addition, the newly available genomic sequence of \textit{Agpsemzm} allows confirmation on the level of intron sizes and sequences that the similarity between \textit{Agpslzm} and \textit{Bt2} is substantially higher than between \textit{Agpsemzm} and either gene. Anchoring of the \textit{Agps} genes on the physical maize map indicates that the genomic regions of \textit{Bt2} and \textit{Agpslzm} show synteny with a single region in rice around \textit{OsAgps2}, a gene exhibiting alternative splicing and targeting to both the cytoplasm and plastid (Lee et al., 2007). On the other hand, \textit{Agpsemzm} shows synteny with \textit{OsAgps1}, which is targeted exclusively to the plastid (Lee et al., 2007).

The comparison of the remaining genomic sequences of \textit{Bt2}, \textit{Agpslzm}, and \textit{Agpsemzm} reveals a conserved structure composed of 10 exons and nine introns. All three genes share the peculiarity that the last intron is situated beyond the coding sequence in the 3’-UTR. Consequently, it is not surprising that the sequence similarity in exon 10 (52% to 81%) is considerably lower than in all preceding exons (81% to 97%), with the exception of exon 1. Sequence similarity is highest in exons 5 and 6, hinting possibly at some functionally important features in this part of the protein in addition to the four well-characterized domains, ATP site (junction exon 2/exon 3), catalytic site (exon 3), Glc-1-P site (exon 3), and activator site (exon 9; Crevillen et al., 2003).

\textbf{Influence of bt2-H2328 on the Expression of Genes Involved in Polysaccharide and Amino Acid Metabolism}

A comparison of the transcriptome of \textit{bt2-H2328} and wild-type kernels revealed a widespread influence of the mutation on transcription because it affected 13% of the probe sets present on the array. Expression differences at 35 DAP concerned primarily genes involved in polysaccharide or amino acid metabolism, but not in lipid or protein metabolism. Whereas the 18K Affymetrix chip admittedly does not represent the entire maize genome and many of the probe sets present on the chip do not carry an annotation allowing a link to GO terms, the data nevertheless strongly support the idea that a block in starch synthesis triggers adjustments on the transcriptional level that favor the flow of excess carbon into glycolysis, TCA, and amino acid synthesis.

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
Metabolite & Wild Type & Mutant & $P$ Value$^a$ & Ratio Mutant/Wild Type \\
& $\mu$g/mg dry wt & $\mu$g/mg dry wt & & \\
\hline
Citrate & 24.96 ± 1.86 & 66.97 ± 4.64 & 2.00E-09 & 2.68 \\
Choline & 1.04 ± 0.11 & 1.68 ± 0.25 & 1.60E-04 & 1.62 \\
Suc & 108.93 ± 18.62 & 154.14 ± 11.76 & 5.14E-04 & 1.42 \\
Pro & 13.60 ± 1.59 & 16.82 ± 1.76 & 7.75E-03 & 1.24 \\
Lipid 4.9$^b$ & 32.87 ± 2.35 & 26.19 ± 1.88 & 2.86E-04 & 0.80 \\
M 4.5$^c$ & 20.92 ± 2.65 & 14.36 ± 1.40 & 3.21E-04 & 0.69 \\
\hline
\end{tabular}
\caption{Metabolite levels in wild-type and bt2-H2328 embryos at 35 DAP}
\end{table}

*a t test of biological triplicate and technical replicate; only metabolites with $P$ value <0.01 are shown. $^b$Unidentified lipid with peak at 4.9 ppm. $^c$Unidentified compound with peak at 4.5 ppm.
A more precise dissection of the metabolic pathways that are turned on or off in mutant kernels is hampered by several factors. (1) In many cases, it is impossible to determine the subcellular localization of the encoded enzymes because the probe sets on the array are frequently based on EST assemblies that only partially cover a given gene; (2) only in rare cases is information available on the expression pattern of a given gene in the different compartments of the maize kernel; (3) in many metabolic pathways, not all the enzymes involved are represented on the array; (4) most enzymes are encoded by multigene families and individual members do not always show the same trends of expression; and (5) many of the encoded enzymes participate in more than one metabolic pathway and/or can function in two directions. For example, phosphoglucomutase (4 in Fig. 5) is needed in Suc biosynthesis and Suc degradation, in starch synthesis and starch degradation, and can be found both in the cytoplasm and plastids. Consequently, it is difficult to draw any conclusion from the down-regulation of one of the three genes present on the array.

Nevertheless, some information can be gained concerning the fate of Suc. There are three enzymes involved in Suc synthesis and/or cleavage: invertase (IncW, Ivr; 1 in Fig. 5), Suc synthase (SuSy; 2 in Fig. 5), and Suc phosphate synthase (SPS; 6 in Fig. 5). Among the six characterized maize invertase genes (Kim et al., 2000), only IncW1 is up-regulated. IncW1 is expressed in the entire endosperm and is the major invertase transcript found during the filling stage. However, its biological role has been elusive in contrast to the IncW2-encoded invertase, which is expressed only at the base of the endosperm and is involved in Suc unloading (Chourey et al., 2006). Because IncW1 is a cell wall-bound invertase, its overexpression leads to increased, irreversible Suc cleavage in the apoplast and consequently to increased extracellular levels of Glc and Fru. Among the three characterized SuSy genes (Carlson et al., 2002), only Sus1 is down-regulated in bt2-H2328 kernels. Sus1 activity in the kernel is smaller than that of Sh1 and both enzymes are thought to contribute mainly to cellulose, rather than starch, biosynthesis. The concomitant down-regulation of genes encoding UDP-Glc dehydrogenase (3 in Fig. 5) and UDP-glycosyltransferase (7 in Fig. 5) points to a general repression of pathways using Suc for cell wall biosynthesis. Finally, there is contrasting regulation for genes encoding SPS, which is mainly used in leaves to synthesize Suc but has also been found in the maize kernel, where it is supposed to catalyze the resynthesis of Suc in the basal part of the endosperm (Im, 2004). Whereas the expression patterns of seven SPS genes have been reported in maize, the authors make it impossible to relate their proprietary data to public sequences or genes (Lutfiyya et al., 2007). Our own RT-PCR data indicate that the up-regulated gene is a constitutive gene, whereas the two down-regulated genes are primarily expressed in leaves (data not shown). One might speculate that the down-regulation is a feedback mechanism normally operating in the leaf, whereas the induction could be due to a lack of intermediates in cell wall synthesis.

Because knowledge is a lot more limited concerning gene families encoding enzymes in glycolysis, TCA, PPP, and amino acid biosynthesis, it is difficult to discuss individually all the up- and down-regulated genes shown in Figure 5. Nevertheless, some general trends emerge. Only up-regulation is found for genes involved in the PPP and the synthesis of aromatic amino acids, and considerably more up- than down-regulation for genes in the TCA cycle (Fig. 5), whereas the picture is more complex for glycolysis and the synthesis of other types of amino acids. This confirms on the gene level the conclusion of the global analysis, that excess Suc is used for the synthesis of amino acids rather than starch.

In addition, our data corroborate the overall conclusion of two previous, much more limited studies, that there is coordinated transcriptional regulation of storage product genes in the maize endosperm (Doehlert and Kuo, 1994; Giroux et al., 1994). However, whereas the authors of the latter study had found an increase in Sh1, Sus, Sh2, Wx1, Zein (small and large), Agp1, and Agpsemzm genes at 22 and 30 DAP of the bt2-7480 mutant, we found either constant expression (Sh1, Wx1) or decreased expression (Sh2, Zein, Sus, Agpsemzm) at 35 DAP in the bt2-H2328 mutant. These differences may be caused in part by technical differences; our microarray data (15 probes/gene) and RT-PCR data obtained with wild-type and mutant kernels stemming from the same segregating ear were probably more specific and more homogeneous than the previous northern data with wild-type and mutant kernels harvested from sibling plants. Biological explanations include the differences in the bt2 allele, the genetic background, and the developmental stage. Because there are important differences and sometimes inversions in the relative abundance of gene expression between 14, 22, and 30 DAP, one does not necessarily expect similar results at 30 (previous study) and 35 DAP (our study). Finally, our data are easier to reconcile with a decrease in Sh2 protein in bt2 kernels at 30 DAP (Giroux et al., 1994) and a 3-fold decrease in zein content in mature bt2 kernels (Tsai et al., 1978).

Suc is a prime candidate to play a role in the mechanism, which translates the strong reduction of AGPase activity into transcriptional activation or repression because it is strongly accumulated in the bt2-H2328 mutant and known to act as a signal molecule (Borjisjuk et al., 2004; Koch, 2004). Suc response elements have been pinpointed in promoters and trans-acting factors binding to theses cis-elements have been identified (Sun et al., 2003). The transcript levels of Sh1, Sh2, and Bt2 have been shown to vary when maize kernels are exposed to different Suc concentrations (Giroux et al., 1994), and other genes coding for enzymes in carbohydrate or amino acid metabolism may be regulated in the same way. The precise mechanism of the signal transduction downstream of Suc remains to
be elucidated, but two functionally redundant protein kinases have recently been implicated in this pathway in Arabidopsis (Baena-Gonzalez et al., 2007). It would be interesting to assess the role of the orthologous proteins on global transcriptional regulation in the maize kernel.

**Metabolic Adjustments in bt2-H2328 Kernels**

$^3$H NMR metabolomics confirmed several of the predictions based on the transcriptome data of bt2-H2328 kernels. Increased levels of citrate, fumarate, and malate correlate well with up-regulation of genes in the TCA cycle, increased levels of Tyr, Val, and Ala with an overall effect on the transcriptome of amino acid synthesis. In addition, stimulation of glycolysis in bt kernels is corroborated by earlier results in a non-defined bt mutant, which demonstrated a slight increase in Glc-1-P and Glc-6-P, no influence on Fru-6-P or Fru-1,6-bisP, and doubling up in dihydroxyacetone phosphate, Glc-3-P, and Fru-2,6-bisP (Tobias et al., 1992). The accumulation of lactate found in this study may also be indicative of this stimulation. The overall data also fit well with the finding that the bt2 and sh2 mutations, but not other mutations in starch synthesis (incW2, sh1, susl, ae, wx1, or sus1), trigger significantly increased fluxes of carbohydrates through glycolysis, PPP, TCA cycle, and gluconeogenesis (Spielbauer et al., 2006). Increased levels of the TCA cycle metabolites malate and citrate had also been found in transgenic potato tubers with strongly reduced AGPase activity due to an antisense construct of the small subunit gene Agp-B (Geigenberger et al., 1999). This suggests that at least some of the metabolic adjustments made in sink organs with reduced AGPase activity are similar in monocot and dicot.

Surprisingly, the metabolic changes in bt2-H2328 kernels were not restricted to the endosperm, where Bt2 is preferentially expressed, but concerned also the embryo, where the nonaffected Agpsemzm gene is the main contributor to AGPase activity. Because Suc levels are also increased in the embryo, one may hypothesize that part of the excess Suc is transported from the endosperm to the embryo, where it triggers similar metabolic adjustments as in the endosperm. These findings on the metabolic level are new, even though differences between AGPase activities in wild-type and bt2 kernels had not only been established in endosperm but also in the embryo but not the pericarp (Dickinson and Preiss, 1969).

The biological role of other metabolites with increased levels in bt2-H2328 kernels is less straightforward. Choline is primarily used for the synthesis of the vital membrane lipid phosphatidyl choline, but its increase in mutant kernels may rather be linked to its additional role as a precursor for the synthesis of betaine. Maize belongs to the plant species that accumulate betaine in response to high salinity, cold, and drought (Rhodes and Hanson, 1993), and the synthesis of this osmoprotectant could possibly counteract the osmotic stress exerted by high Suc levels. GABA is a nonprotein amino acid that also accumulates in response to biotic and abiotic stresses (Bouche and Fromm, 2004). Its synthesis via the GABA shunt bypasses two steps of the TCA cycles, among them succinyl-CoA ligase (19 in Fig. 5), the only one showing a clear transcriptional down-regulation in bt2-H2328 kernels. This suggests that the TCA cycle not only is activated in mutant kernels, but also that it functions in a modified manner.

**CONCLUSION**

The antagonistic relationship between the starch and protein content of the maize kernel is a well-known phenomenon among maize breeders and global analyses have shown that increases in starch were generally linked to decreases in protein (Duwick and Cassman, 1999). Mutants in starch synthesis are a means to investigate the starch/protein balance. We show here that, in the bt2-H2328 mutant, important changes take place in the transcription of genes involved in amino acid synthesis and that there is an increase in the content of certain amino acids. Whereas at first sight these data could be interpreted as a shift in the type of reserve substances from starch to protein, things are certainly a lot more complex. In fact previous data document that the bt2 mutation causes a strong decrease in zeins, the major storage proteins in the maize kernel, whereas the content of non-zein proteins is increased (Tsai et al., 1978). Taken together with the synthesis of stress-related compounds in mutant kernels, the data suggest that at least some of the transcriptomic and metabolic adjustments reflect a response to an emergency situation rather than a concerted shift to the synthesis of alternative reserve substances.

**MATERIALS AND METHODS**

**Plant Material**

The bt2-H2328 mutant was isolated from a Mu-based mutant collection established by Biogemma SAS (Martin et al., 2006). Wild-type lines used were F252 (gift of A. Charcosset, INRA, Moulon) for back crosses of the mutant collection and A188 (Gerdex and Tracy, 1993) for molecular work. Mutants sh1 (stock 912A), sh2-N391 (stock 318E), bt1-N1992 (stock 515E), bt2 (stock 411A), sus1 (stock 407D), and sus2 (stock 690D) were provided from the Maize Genetics Cooperation Stock Center (University of Illinois). Plants were grown in open air in Lyon (France) or in a greenhouse with a 16-h illumination period at 24°C/19°C (day/night) without control of the relative humidity.

**Allelism Tests**

Homoyzogous mutant plants from the Maize Stock Center (bt1, bt2, sh1, sh2, sus1, sus2) and from our collection (E2416, H0182, H0816, H2328) were grown in the greenhouse. At least two plants of each mutant were crossed with the mutant H2328. In control experiments, at least one plant of each mutant was self-pollinated and at least one plant of each mutant was crossed with inbred line F252.

**Amplification of Mu-Flanking Sequences**

The Bt2 gene of family H2328 was scanned for Mu insertions by PCR reactions with primers Bt2-353 (5′-AATGCTTGGTGTATAGCC-3′)
and Bt2-1649 L (5'-CTCGGGTTCCAGGCAGCTTCG-3') for segment a, Bt2-1649 U (5'-GGGAGTTCAGCTACAC-5') and Bt2-2770 L (5'-TGGACA- AAATCGAAGAGACAGA-3') for segment b, Bt2-2634 U (5'-TCCTG- CTITCCTTCAAGCCGTC-5') and Bt2-3481 L (5'-TCCTTGCGCTCC- TTGG-3') for segment c, and Bt2-3803 U (5'-CGCAAGTCAAAGGATAAA- GATT-3') and Bt2-6404 U (5'-ATGAAAGAAGCCGTGGCATTAGAA-3') for segment d, either as couples or in conjunction with primer AIMS2-Mu2 (5'-GCTCTTCTGCTCATAAAGGCGATTCTC-3').

Cosegregation Analysis

One hundred twenty normal looking kernels (wild type or heterozygous) from one to four ears segregating for the mutation under investigation were sown in the field. After germination, leaf samples were taken from each plant for gDNA extraction and genotyping. The following primers were used either as a couple (band in wild-type and heterozygous plants) or in combination with the Mu-specific primer AIMS2-Mu2 (band only in heterozygous plants): H6P-L634cos3 (5’-TGGCGCCAAAGAGCAGAATCGT-3’) and H6P-L634cos4 (5’-GTTCGAAAGGAGCCACTG-3’) for the insertion in H6P, H6P-L635cos1 (5’-TGGCCGATATCGCATACAGT-3’) and H6P-L635cos2 (5’-TATTGACGTATGCTCCGTTCT-3’) for the insertion in H6P, and Bt2-S5 (5’-CAATACAGATTGTAGATGTT-3’) and Bt2-S4 (5’-GCTTACCTTAAATAAACACATCTC-3’) for the insertion in Bt2. At flowering, all the plants were selfed for phenotyping. Fully wild-type ear and segregating mutant ear were indicative of a wild-type plant and a heterozygous plant, respectively.

Cytological Analysis

Kernels were harvested at 30 DAP and cut into three pieces of equal width along the longitudinal axis. The central slice was fixed in paraformaldehyde, dehydrated, embedded in paraffin, sectioned, and stained as described previously (Ospahl-Ferstad et al., 1997). The periodic acid-Schiff procedure stains insoluble carbohydrates, including starch and cellulose, in pink (Vozzo and Young, 1975).

Genetic Mapping

The H6P locus was mapped by PCR with primers H6P-L634cos3 and H6P-L634cos4 (see section on cosegregation analysis) in the intermated B73/Mo17 mapping population (Lee et al., 2002) exploiting a size polymorphism between the parental lines B73 and Mo17.

Agps Reference Sequences

The analysis of genomic sequences was based on a B73 bacterial artificial chromosome (BAC) sequence for B2 (AC193357), which fully included the previously published B73 sequence (AF334959), and B73 BAC sequences for Agpsezm (AC177860) and Agpslzm (AC1209218). A 46-bp 5’-UTR was defined for B2 by similarity with EST CO455578. The full-length cDNA sequences EC960331 and DR788076 as well as EST contigs B7106913 and AY105913 were the basis for a 140-bp 5’-UTR and a 245-bp 3’-UTR of Agpslzm. A 130-bp 5’-UTR and a 233-bp 3’-UTR were defined for Agpslzm based on full-length cDNA sequences DR969809 and DR969810. The flanking sequence tags of the linked insertions H6P and H6P were given the accession numbers EU137670 and EU137671, respectively.

RT-PCR

Total RNA was isolated using the following procedure. Tissues were ground to powder under liquid nitrogen and transferred to a tube containing equal volumes of extraction buffer (200 mM Tris-HCl, pH 9, 400 mM KCl, 200 mM Suc, 35 mM MgCl2, 25 mM EGTA) and phenol/chloroform (pH 8) and vortexed for 30 s. The aqueous phase resulting from a 5-min centrifugation at 18,000 g was reextracted twice with phenol/chloroform. RNA was precipitated by addition of 1.5 times acid (1/10 volume) and ethanol (2.5 volumes). The RNA pellet was washed with 3 x sodium acetate (pH 6) and resuspended in water. A second acetic acid/ethanol precipitation was performed before final resuspension in water.

RNA was treated with RNase-free DNase and the DNase inactivated according to the instructions of the supplier (Ambion). The RNA was quantified in a spectrophotometer at 260 nm. Approximately 5 μg of total RNA were reverse transcribed using random hexamers (Amersham Pharmacia Biotech) and reverse transcriptase without RNaseH activity (Fermentas) in a final volume of 20 μL. Then 2.5 x 105 copies of GeneAmplifier pAW109 RNA (Applied Biosystems) were added to the RT reaction. The cDNA was diluted 50 times and 5 μL used for amplification by PCR in a volume of 20 μL.

The following primers were used for gene-specific amplification: Bt2-cDNA-F2 (5’-CTGGTTTCTATCGTTCCAGA-3’) and Bt2-cDNA-R3 (5’-CACAGCTCAGTCGCCGTTCTA-3’) for Bt2, leaf-cDNA-F (5’-GCTGACAAGAATTCTCTTGCGAATTT-3’) and leaf-cDNA-R2 (5’-CTGGCTTTCATGTCGCCAATCC-3’) for Agpslzm, and BEF2 (5’-TATCCACGGAGGAAGGCG-3’) and BER2 (5’-AGGACATCTTCTACATC-3’) for Agpslzm. The specificity of the primers was shown by cloning PCR products in tissues with low relative expression (B2 in leaf, Agpslzm in 35-DAP kernel, Agpslzm in 37-DAP kernel) and sequencing of 10 clones per gene. In all three cases, all 10 clones were identical to the reference sequences AF334959 (B2f), AF334960 (Agpslzm), or AC177860 (Agpslzm). The alternatively spliced 5’ ends of B2 were detected with primers Bt2-F21 (5’-GCCCTGTC- AAATGATTCAACATACC-3’) and Bt2a and primer Bt2-F11 (5’-ATGGCGCC- GATAACCTCTGACT-3’) for Bt2b, both in conjunction with the same reverse primer Bt2-R11 (5’-GCCAGCTCTGCCGCTTTTG-3’). The primer Bt2-F11 had been designed to hybridize only to Bt2b and not to Agpslzm, and this specificity was demonstrated by sequencing 10 cloned PCR products obtained with the combination Bt2-F11/Bt2-R11 in both 2DAP kernels and leaves. All 20 sequences corresponded to the B2 reference sequence.

Primers AIMS2-Mu2 (see above) and Bt2-cDNA-R3 (see above) allowed amplification of the b2-H2328 allele downstream of the Mu insertion. Wild-type, +/b2-H2328, and b2-H2328/b2-H2328 plants were identified both by PCR genotyping and by self-pollination followed by phenotypic analysis of the ears. Genetic contamination was shown to be negligible by the use of intron-spanning control primers ART1 (5’-GTCAACTTCTGCCTGACAA-3’) and ART4 (5’-CCGTCGCCACGCTGACAC-3’) amplifying ZmOCL (Khaled et al., 2005).

Leaf RNA was constitutively isolated 18S rRNA gene (primers 5’-CCATCCCTT- CCAGAATTCGAAAGGCCACT-3’) and 5’-CCGTTGCGCAGCCGTTCTA-3’) was used as an internal control of RNA quantity and GeneAmplifier pAW109 RNA (primers 5’-CATGCTACAACTTCTGACTGTA-3’) and 5’-TGACCACCAAGGCGTCTAT-3’) as positive control of the RT-PCR efficiency. To get semiquantitative results, the number of cycles of the PCR reactions was adjusted for each gene to obtain barely visible bands in agarose gels. Aliquots of the PCR reaction were loaded on agarose gels and stained with ethidium bromide.

Transcriptome Analysis

Whole kernels (pericarp + endosperm + embryo) without glumes from greenhouse-grown ears of heterozygous, self-pollinated plants were collected at 35 DAP and visually divided into pools of phenotypically normal looking kernels (genotype +/- or +/-/b2-H2328) and pools of phenotypically mutant kernels (genotype b2-H2328/b2-H2328). Two different ears were used for a biological duplicate. Total RNA was extracted using the following procedure. Samples were ground to powder under liquid nitrogen and transferred to a tube containing 4.5 mL of extraction buffer (0.1 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 1% SDS) and 3 μl phenol/chloroform (pH 8.0), and agitated for 10 min. The aqueous phase resulting from a 20-min centrifugation at 5,000 g was reextracted with phenol/chloroform. RNA was precipitated by addition of 3 μl sodium acetate, pH 5.2 (1/10 volume), and ethanol (2.5 volumes). The RNA pellet was resuspended in water. Contaminating DNA was removed using a DNase set (Qiagen) and RNA purified using the Min Elute kit (Qiagen). All RNA samples were checked for their integrity on the Agilent 2100 bioanalyzer according to the protocols of Agilent Technologies.

Two micrograms of total RNA were used to synthesize biotin-labeled cRNAs with the one-cycle cDNA synthesis kit (Affymetrix). SuperScript II reverse transcriptase and T7-oligo(dT) primers were used to synthesize single-stranded cDNA at 42°C during 1 h, followed by synthesis of double-stranded cDNA using DNA ligase, DNA polymerase, and RNaseH during 2 h at 16°C. After cleanup of the double-stranded cDNA with the Sample Cleanup Module (Affymetrix), in vitro transcription was performed in the presence of biotin-labeled UTP using the GeneChip IVT labeling kit (Affymetrix). The labeled cRNA was purified with the Sample Cleanup Module (Affymetrix) and quantified with Ribogreen RNA quantification reagent (Turner Biosystems). Fragmentation of 15 μg of labeled cRNA was carried out for 35 min at 94°C.
followed by hybridization during 16 h at 45°C to Affymetrix GeneChip Maize Genome Array representing approximately 14,850 maize transcripts, corresponding to 13,339 genes. After hybridization, the arrays were washed with two different buffers (stringent: 6× SSPE, 0.1% Tween 20; and nonstringent: 100 mM MES, 0.1 M Na+, 0.01% Tween 20) and stained with a complex solution including Streptavidin R-Phycoerythrin conjugate (Molecular Probes) and anti-Streptavidin biotinylated antibody (Vectors Laboratories). The washing and staining steps were performed in a GeneChip Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip maize genome arrays were finally scanned with the GeneChip Scanner 3000 7G piloted by GeneChip Operating Software. All these steps were performed on Affymetrix platform at INRA-Urgv in Evry.

The raw CEL files were imported in the Bioconductor software package in R for data analysis (Gentleman et al., 2004). The data were normalized with the gcyra algorithm (Irizarry et al., 2003) available in the Bioconductor package. To determine differentially expressed genes, we performed a usual two-group t test that assumes equal variance between groups. The variance of the gene expression per group is a homoscedastic variance, where genes displaying extremes of variance (too small or too large) were excluded. The raw P values were adjusted by the Bonferroni method, which controls the familywise error rate (Dudoit et al., 2003). A gene is declared differentially expressed if the Bonferroni P value < 0.05. All raw and normalized data are available from the Gene Expression Omnibus repository at the National Center for Biotechnology Information (Barrett et al., 2007) under accession number GSE7030. Overrepresentation of GO terms among differentially expressed genes was assessed with the GO Browser tool of the Functional Genomics suite of the Spotfire Decision Site program (Spotfire). GO annotations of the probe sets were downloaded from the NetAffx Web site (http://www.affymetrix.com; Liu et al., 2003).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Representative quantitative 1H NMR (500 MHz) profiles of extracts of wild-type embryo (A), endosperm (B), or whole kernel (C).

Supplemental Figure S2. Comparison of representative wild-type (A) and bt2-H2328 mutant (B) 1H NMR (500 MHz) profiles of embryo extract.

Supplemental Figure S3. Comparison of representative wild-type (A) and bt2-H2328 mutant (B) 1H NMR (500 MHz) profiles of endosperm extract.

Supplemental Figure S4. PCA of 1H NMR signatures of endosperm extracts of wild type and bt2-H2328 mutant.

Supplemental Figure S5. PCA of 1H NMR signatures of embryo extract of wild type and bt2-H2328 mutant.

Supplemental Table S1. Metabolite levels in wild-type and bt2-H2328 kernels at 35 DAP.

Supplemental Table S2. Metabolite levels in wild-type and bt2-H2328 endosperms at 35 DAP.

Supplemental Table S3. Metabolite levels in wild-type and bt2-H2328 embryos at 35 DAP.

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