Genetic and Physiological Analysis of Germination Efficiency in Maize in Relation to Nitrogen Metabolism Reveals the Importance of Cytosolic Glutamine Synthetase

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We have developed an approach combining physiology and quantitative genetics to enhance our understanding of nitrogen (N) metabolism during kernel germination. The physiological study highlighted the central role of glutamine (Gln) synthetase (GS) and Gln synthesis during this developmental process because a concomitant increase of both the enzyme activity and the amino acid content was observed. This result suggests that Gln is acting either as a sink for ammonium released during both storage protein degradation and amino acid deamination or as a source for amino acid de novo synthesis by transamination. In the two parental lines used for the quantitative genetics approach, we found that the increase in Gln occurred earlier in Io compared with F_2, a result consistent with its faster germinating capacity. The genetic study was carried out on 140 F6 recombinant inbred lines derived from the cross between F_2 and Io. Quantitative trait locus mapping identified three quantitative trait loci (QTLs) related to germination trait (T50, time at which 50% of the kernels germinated) that explain 18.2% of the phenotypic variance; three QTLs related to a trait linked to germination performance, kernel size/weight (thousand kernels weight), that explain 17% of the phenotypic variance; two QTLs related to GS activity at early stages of germination that explain 17.7% of the phenotypic variance; and one QTL related to GS activity at late stages of germination that explains 7.3% of the phenotypic variance. Coincidences of QTL for germination efficiency and its components with genes encoding cytosolic GS (GS1) and the corresponding enzyme activity were detected, confirming the important role of the enzyme during the germination process. A triple colocalization on chromosome 4 between *gln3* (a structural gene encoding GS1) and a QTL for GS activity and T50 was found; whereas on chromosome 5, a QTL for GS activity and thousand kernels weight colocalized with *gln4*, another structural gene encoding GS1. This observation suggests that for each gene, the corresponding enzyme activity is of major importance for germination efficiency either through the size of the grain or through its faster germinating capacity. Consistent with the possible nonoverlapping function of the two GS1 genes, we found that in the parental line Io, the expression of *Gln3* was transiently enhanced during the first hours of germination, whereas that of *gln4* was constitutive.

Seeds are complex structures consisting anatomically and genetically of three parts: the embryo, the endosperm, and the seed coat. During their development, seeds are supplied by the mother plant with assimilates. From a physiological point of view, seeds behave as sink organs accumulating C and N metabolites stored as starch, proteins, amino acids, or lipids. C and N metabolism in developing seeds has been extensively studied in various monocot species, i.e. maize (*Zea mays*) and wheat (*Triticum aestivum*), as well as dicot species, i.e. pea (*Pisum sativum*), soybean (*Glycine max*), and alfalfa (*Medicago sativa*; Weber et al., 1997; Baskin and Baskin, 1998). Maturation, which corresponds to the final stage of seed development, is characterized by a dehydration phase, which leads to a loss of more than 90% of its water content and a dramatic decrease in its metabolic activity (Baskin and Baskin, 1998). In contrast, the induction of germination after rehydration of the seed induces an increase in both respiration and metabolic activity thus allowing the mobilization of C and N reserves. The control of the mobilization of C reserves during germination has been extensively studied through the regulation of α-amylase expression in barley (*Hordeum vulgare*) aleurone cells, emphasizing the role of phytohormones such as gibberellins and abscisic acid (ABA) during programmed cell death (Fath et al., 2000; Bethke and Jones, 2001; Gomez-Cadenaz et al., 2001). In contrast, mobilization of N reserves has received much less attention, although several earlier studies have pointed out the need for amino acid inter-conversion. In germinating seeds, the spectrum of amino acid released and transported after storage protein hydrolysis is not complete and therefore does not allow cytoplasmic proteins to be synthesized (Lea and Joy, 1983). It has also been proposed that amino acid

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catabolism occurring during the early stages of germination is a source of energy and of nutrients for expanding new tissue. Supply of amino acid is therefore of major importance in the control of germination (Below et al., 2000) and may be controlled by the phytohormone ABA (Garciarrubio et al., 1997).

A number of enzymes of amino acid catabolism in germinating seeds, such as Pro dehydrogenase and arginase, are regulated either at the transcriptional or the posttranscriptional level, for an optimal control of their relative activity during specific phases of the germination process (Nakashima et al., 1998; Goldraij and Polacco, 1999, 2000). Both the mobilization of seed storage proteins and the oxidative deamination of free amino acids lead to a release of ammonium (Goldraij and Polacco, 1999, 2000). The released ammonium can represent 50% of the N made available during seed germination (Garciarrubio et al., 1997). Ammonium is further recycled into Glu to generate Gln through the reaction catalyzed by the enzyme Gln synthetase (GS). In higher plants, GS is represented by two different isoforms located either in the cytosol (GS1) or in the plastids (GS2). It has been proposed that each GS isoenzyme plays a specific role during plant growth and development in the assimilation or re-assimilation of ammonia originating from a wide variety of anabolic or catabolic processes (Cren and Hirel, 1999). For example, during germination of barley seed, an increase in GS1 was observed both in the scutellum and aleurone cell layer (Marttila et al., 1993). The transient induction of a seed-specific GS isoenzyme was also reported during the imbibition of sunflower (*Helianthus annuus*) seeds (Haba et al., 1992). Although this has never been fully demonstrated, it has been proposed by several authors that each cytosolic GS isoenzyme plays a specific physiological role suited to the physiological need of the plant (Woodall et al., 1996; Cren and Hirel, 1999). This may explain why seed-specific GS1 isoenzymes are differentially expressed during certain phases of seed development (Rastogi et al., 1998).

In light of these previous studies, we have investigated further the role of the enzyme GS during maize kernel germination by combining a physiological and a genetic approach. The physiology of germination was first investigated by monitoring in parallel the evolution of various N metabolites and the changes in the activity of key enzymes involved in C and N metabolism during the early stages of germination in two genetically distinct lines of maize. A population of maize recombinant inbred lines (RILs; Causse et al., 1996) produced from the crossing of the two lines used for the physiological studies was then used for a quantitative genetic approach. This approach showed that quantitative trait loci (QTLs) related to germination efficiency, QTLs related to grain size/weight, and QTLs for GS activity during germination colocalized on a genetic map of maize. We also found that some of these QTLs also colocalized with two of the GS1 structural genes, *gln3* and *gln4*, located on chromosomes 4 and 5, respectively, strengthening the idea that the corresponding enzyme activity is likely to play a major role in the control of kernel germination.

RESULTS

Physiological Characterization of N Metabolism during Seed Germination in Two Maize Genotypes Io and F2

Preliminary experiments were conducted to determine whether the germinating properties of two maize genotypes (Io and F2) used to produce the population of RILs were different. The physiology of the seeds of the two genotypes was then further characterized by monitoring the evolution of marker enzyme activities and metabolites representative of C and N metabolism and source/sink transition during the early germination events.

Compared with F2, the parental line Io was characterized by kernels of both smaller size and weight that germinated faster. To determine kernel size accurately, kernels were weighed, and the result was expressed as thousand kernels weight (TKW). The speed of germination was determined at two temperatures, 21°C and 25°C, by measuring the T50 representing the rehydration time at which 50% of seeds germinate. As shown in Figure 1, germinating the seeds at either 25°C or 21°C resulted in a increase in the T50 of F_2 of 11 and 12 h compared with Io. Decreasing the temperature from 25°C to 21°C slowed down germination speed in the same manner in both genotypes, which resulted in a delay of the T50 by about 28 h (Fig. 1). In both genotypes, we also observed 100% of the seeds germinate regardless of the temperature conditions. These two observations demonstrate that the differences observed for the T50 between the two genotypes were physiologically and/or genetically relevant and were not attributable to any damage or aging effect of the kernel. Because all of the physiological experiments conducted in parallel at the two temperatures 25°C and 21°C gave similar results (data not shown), only the series of experiments performed at 25°C will be presented.

Variation in seed total free amino acid content was monitored during the early germination events. In both genotypes, a large increase in amino acids was observed a few hours after the beginning of kernel imbibition. This increase was faster for the genotype Io (Fig. 2), consistent with the previous finding that this genotype germinates faster. The increase also corresponded with an increase in most of the major amino acids of the seed, Gln, Pro, and Asn being the most abundant. Asn was proportionally more abundant in the early phases of germination, whereas Gln and Pro became predominant during the later stages of germination (Fig. 2). The rate of accumulation of all individual amino acids was always faster for Io.

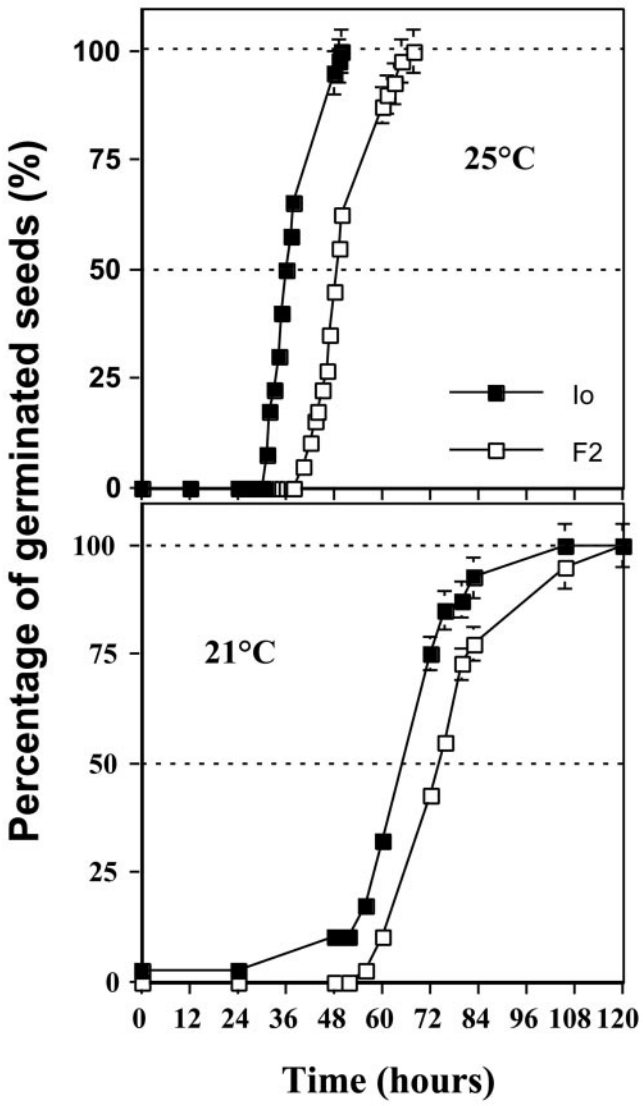

Figure 1. Germination efficiency of two maize lines Io and F_2 determined as percentage of germinated kernels at various times after the beginning of imbibition at two temperatures, 21°C and 25°C. The results are the mean of three replicates of 40 grains germinating in separate petri dishes.

After 60 h, Gln represented about 30% of total amino acids, followed by Pro and Asn each representing approximately 15% of the total.

As shown in Figure 3, the free ammonium content in both parental lines was relatively constant until 40 h of germination. After 40 h, an accumulation of ammonium was observed, which was slightly higher in Io compared with F2.

The results of metabolites analysis during germination prompted us to further investigate the evolution of GS, GOGAT, and Glu dehydrogenase (GDH) activities, three enzymes potentially involved in the synthesis and recycling of inorganic and organic N. In both parental lines, GS activity exhibited a 4-fold increase during germination (Fig. 4). This increase correlated with the increase in Gln content. A similar increase in both NADH-dependent GOGAT and aminating GDH activity was also observed (Fig. 4). A low ferredoxin (Fd)-GOGAT activity was detected during germination, but its contribution was negligible compared with its pyridine nucleotide counterpart (data not shown). Interestingly, no GDH-deaminating activity was detected.

During germination, phospho*enol*pyruvate carboxylase (PEPc) activity remained practically constant except during the first 24 h in which a slight increase in the enzyme activity was observed in the genotype Io (Fig. 4). Because PEPc activity was also not significantly different between Io and F2, it can therefore be considered as a constitutive control in comparison with the activities of GS, GOGAT, and GDH.

Differences observed between both genotypes in terms of germination efficiency (T50) and amino acid metabolism could be attributable to differences in speed and/or efficiency of rehydration, which is usually related to testa structure rather than to metabolic activity. To check this hypothesis, Io and F_2 kernels were rehydrated at 4°C for 24 h before being transferred to 25°C. Such treatment allows metabolic activity to start only when the kernels have reached the same water content. After incubation at low temperature, both in Io and F2, T50 was delayed by 15 to

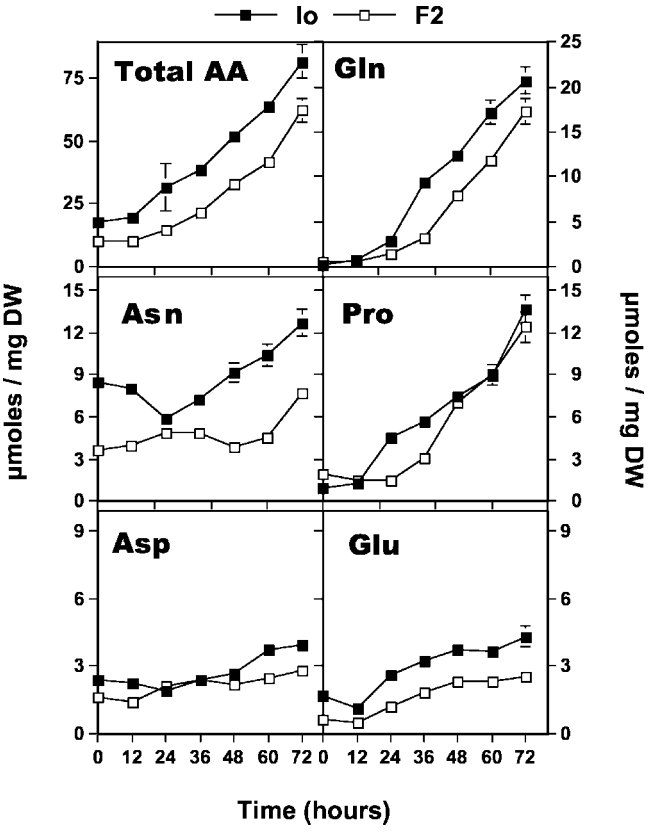

Figure 2. Variation in total and major amino acid contents in kernels of two maize lines Io and F_2 germinating at 25°C. The results are the mean of three replicates ± SE ($P < 0.05$).

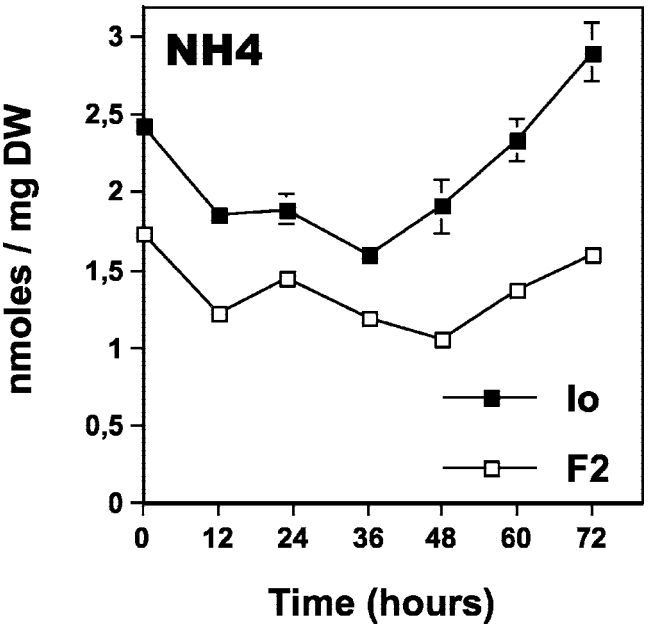

Figure 3. Variation in free ammonium contents in kernels of two maize lines Io and F_2 germinating at 25°C. The results are the mean of three replicates ± SE ($P < 0.05$).

17 h, respectively, compared with a germination performed directly at 25°C. Interestingly, the increase in GS activity was delayed accordingly, whereas PEPc activity was already active, even after low temperature treatment (data not shown).

Genetic Analysis and Mapping of QTLs for Biochemical and Germination Traits

We have developed a quantitative genetic approach during seed germination similar to that used to better understand the genetic and physiological basis of N use efficiency in developing maize plants (Hirel et al., 2001). We have studied in parallel the developmental traits related to germination efficiency (T50) and kernel size/weight (TKW) and the biochemical traits related to N assimilation and recycling (GS and GDH activities) for the detection of QTLs. Coincidences between QTLs for biochemical traits related to N assimilation and recycling and QTLs for germination and kernel size/weight traits will give a physiological meaning to the QTLs for developmental traits. This investigation will thus allow us to determine whether enzymes involved in Gln synthesis and recycling may play an important function in germination efficiency by controlling T50 and TKW, a trait dependent on resource allocation that is related to germination efficiency (C. Rouillon and A. Limami, unpublished data). Furthermore, this work will help us unravel the relationship between kernel size/weight and germination efficiency.

During germination at 25°C, TKW, T50, GS, and GDH activities were measured using a population of 140 RILs derived from the cross between Io and F_2 (Causse et al., 1996) and used both for the determination of correlation between various studied traits and for the localization of QTLs. The characters TKW, T50, and GS activity exhibited a normal distribution and transgression in both directions, indicating that they are under the control of a large number of genes, which is a characteristic of polygenic traits (data not shown). GDH activity data failed to fit a normal distribution. QTLs detected for both germination and biochemical trait GS activity are presented in Table I. The position of the different QTLs on the maize RFLP map is shown in Figure 5.

QTLs for GS Activity and Germination Traits T50 and TKW

Three QTLs for seed GS activity were detected (Fig. 5). Two of them corresponded to GS activity measured 48 h after the beginning of imbibition, one being located on chromosome 4 (130 centiMorgans [cM] near the marker sc419) with the favorable allele from parental line F2, whereas the other was located on chromosome 5 (182 cM near the marker scd258A) with the favorable allele from parental line Io. These two QTLs together explained 17.7% of the phenotypic variation.

Interestingly, the QTL for GS activity detected on chromosome 4 coincided with the *gln3* GS structural gene, whereas the other found on chromosome 5 coincided with the *gln4* GS structural gene. Another QTL for GS activity measured at 72 h after the beginning of imbibition was localized on chromosome 1 at 104 cM near the marker sc145 with the favorable allele from parental line Io.

No QTLs for GDH activity were detected. The presence of QTLs for NADH-GOGAT activity was not investigated because of the difficulty in accurately measuring its activity in vitro on such a large number of individuals.

Three QTLs for T50 that explain 18.2% of the phenotypic variation were identified (Fig. 5). Two of these QTLs were located on chromosome 4 (114 and 204 cM near the markers sc59C and umc133, respectively), both with the favorable allele from the parental line F2. The third QTL was located on chromosome 2 (84 cM near marker sc199) with the favorable allele from the parental line Io.

Three QTLs for TKW of the RILs kernels that explain 17.0% of the phenotypic variance were detected (Fig. 5). Two of these QTLs were mapped on chromosomes 5 at positions 106 and 170 cM near the markers sc343B and sc258A, both with favorable allele from parental line Io. The third QTL was mapped on chromosome 4 at position 206 cM near marker umc133 with favorable allele from parental line F2.

Correlation between Physiological and Germination Traits and Colocalization of QTLs

GS activity during the late stages of germination (72 h) showed a highly significant ($P < 0.0001$) neg-

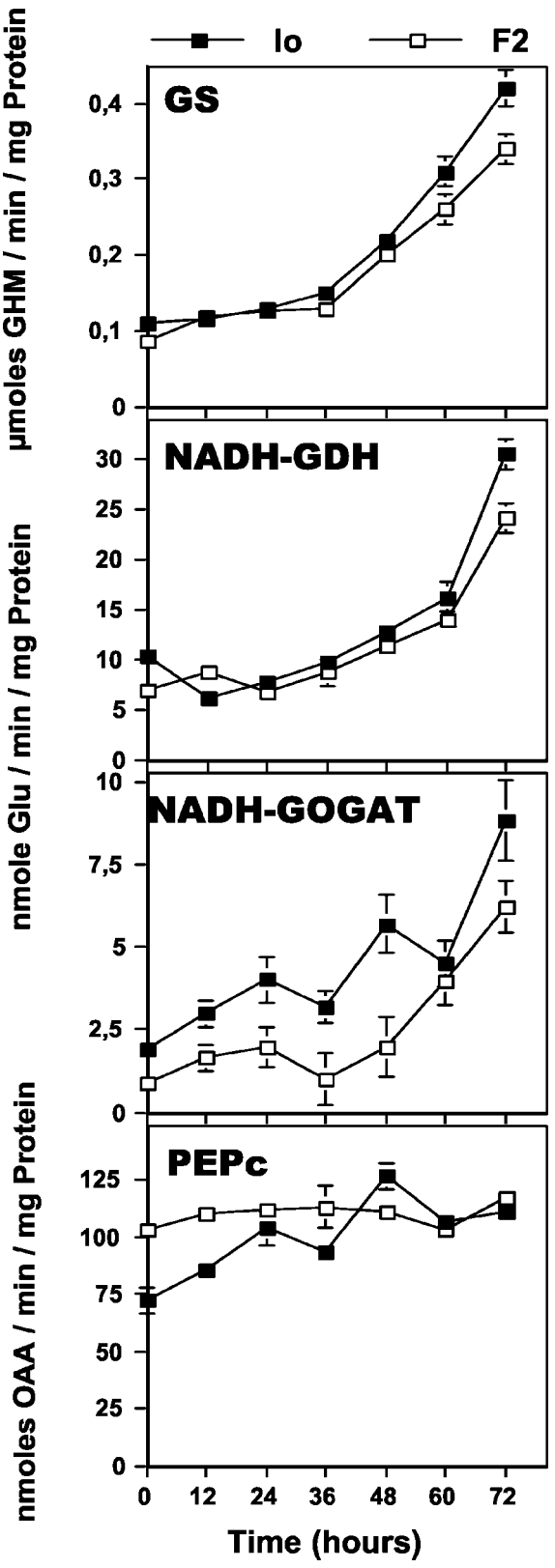

Figure 4. Variation in enzymes activities throughout germination process in kernels of two maize lines Io and F_2 at 25°C. NADH-GDH, GDH tested for its oxoglutarate-aminating activity; NADH-GOGAT, Gln oxoglutarate amino transferase. The results are the mean of three replicates ± SE ($P < 0.05$).

ative correlation ($r = -0.30$) with T50. It can be hypothesized therefore that GS activity is related to the germination process and is likely to be a determinant of the germination efficiency. GS activity showed also a highly significant ($P < 0.005$) negative correlation ($r = -0.25$) with TKW, whereas this trait showed a highly significant ($P < 0.001$) positive correlation ($r = 0.29$) with the trait T50, indicating that the efficiency of maize germination could be related to the size/weight of the kernel. Together, these correlation studies suggest that kernels of small size (low TKW) exhibiting a faster and therefore a more efficient germination (low T50) contain a higher level of GS activity.

An interesting finding in this study is the positive coincidence of two QTLs for GS activity and the germination trait T50 and kernel size/weight TKW. One QTL for GS activity located on chromosome 4 (*gln3* locus) coincided with a QTL for T50. This coincidence is consistent with our hypothesis that GS would play a major role during germination. On chromosome 5, the other QTL for GS activity (*gln*4 locus) was coincident with a QTL for TKW. It is interesting to note that Hirel et al. (2001) also found a colocalization at the *gln*4 locus with several QTLs for physiological traits such as leaf GS and NR activities and agronomic traits such as TKW and kernel yield. The two QTLs for the germination trait T50 and kernel size/weight TKW located on chromosome 4 were coincident near the marker umc133, thus confirming the interaction of the size/weight of the kernel with its efficiency of germination.

Expression of Genes Encoding Cytosolic GS during Maize Kernel Germination

The expression of the five genes (*gln1–gln5*) encoding cytosolic GS during maize kernel germination was monitored in the parental line Io using specific cDNA probes (Sakakibara et al., 1992b). Results of northern-blot analysis showed that only *gln3* and *gln4* were expressed, whereas no expression was detected for the three other members of the multigene family (Fig. 6). *Gln4* was constitutively expressed over 72 h after the beginning of imbibition, whereas *gln3* was transiently expressed with a sharp increase in the corresponding mRNA level during the first 24 h of germination, followed by a decrease during the next 36 h. Similar results were obtained with the parental line F_2 (data not shown).

DISCUSSION

Physiological Characterization of N Metabolism during Maize Kernel Germination

The induction of germination by rehydrating the seeds leads to an increase in respiration and metabolic activity that allows C and N reserves to be mobilized (Nozillo et al., 1983). The activation of

Table I. *QTLs for GS activity at early (48 h) and late stages of germination (72 h) and traits related to grain germination efficiency (T50) and grain size/weight (TKW)*

QTLs detected by simple interval mapping. Distance cM, position at which the QTL has been mapped; cM distance between the QTL and the closest marker on the genetic map; r^2, Percentage of phenotypic variance explained by the QTLs. Confidence interval is for LOD −1; Additive effect corresponds to one-half of the difference between the estimated value of the two homozygous genotypes at the QTL. Positive and negative values for additive effect by convention mean that favorable allele is from parental line Io and F_2, respectively.

Trait	r^2	Chromosome	Location			Confidence Interval	LOD	Additive Effect	Favorable Allele from Parental Line
			Marker	+cM	Distance				
		no.			*cM*				
Early GS activity	17.7	4	sc419	+0	130	122–134	3.11	−0.009	F_2
		5	sc258A	+13	182	166–214	2.64	+0.012	Io
Late GS activity	7.3	1	sc145	+17	104	88–138	2.08	+0.022	Io
T50	18.2	2	sc199	+2	84	78–94	2.53	+5.728	Io
		4	sc59C	+10	114	104–126	2.17	−5.271	F_2
		4	umc133	+25	204	178–226	3.42	−14.597	F_2
TKW	17.0	4	umc133	+27	206	178–234	2.40	−38.298	F_2
		5	sc343B	+1	106	98–118	2.93	+18.320	Io
		5	sc258A	+2	170	156–186	2.80	+18.986	Io

metabolism is well illustrated in our study by the high PEPc activity that remained relatively unchanged throughout the germination process. This activity was independent of the genotype and germination condition, i.e. rehydration temperature (4°C, 21°C, or 25°C). Interestingly PEPc was active even in dry kernels, and the activity was comparable with that observed in rehydrated seeds. Previous studies have also shown a relatively high PEPc activity during the germination process (Gonzalez et al., 1998), consistent with the role of this enzyme in providing, through its anapleurotic function, C skeletons to the tricarboxylic acid cycle. This metabolic pathway allows, in turn, the synthesis of 2-oxoglutarate for the GS/GOGAT cycle and the fueling up of the respiratory chain for the production of energy.

In contrast, the changes in both N metabolite concentrations and enzymes activities related to N assimilation and recycling illustrate the induction of a de novo metabolic activity induced very early after the onset of rehydration. These metabolic changes were characterized by an increase in total amino acid, particularly Gln and ammonium. Moreover, we observed a shift in the composition of the amino acid pool from a storage profile, in which Asn was the most abundant, to an anabolic profile, in which the Asn content decreased whereas that of Gln, Glu, and Pro become predominant. In particular, our results highlight the central role of Gln during germination of maize kernel probably acting as a sink for ammonium released during both storage protein degradation and amino acid deamination. For example, activities of enzymes involved in amino acid deamination and oxidation in seeds such as asparaginase (Lea and Joy, 1983) and arginase and urease (Goldraij and Polacco, 1999, 2000) are known to be developmentally controlled. They are induced during germination while in developing embryos, a regulatory mechanism impedes amino acid breakdown (Goldraij and Polacco, 1999, 2000). Furthermore, we found that the increase in Gln occurred earlier in Io compared with F2, a result consistent with its faster geminating capacity revealed by the T50 of the two parental lines. It is likely that the increase in soluble Pro during germination was the result of the degradation of storage proteins, which generally have a high Pro content (Lea and Joy, 1983). However, it cannot be completely excluded that the increase in this amino acid is attributable to de novo synthesis as the result of a transient stress during the first stages of rehydration similar to that observed during salt and drought stress (Farrant et al., 1989; Schwab et al., 1989). Although our knowledge about the precise route of Pro synthesis in germinating seedlings is limited, its increase during seed germination has been reported in several species (Lea and Joy, 1983). Pro is directly linked to Glu metabolism (Delauney and Verma, 1993) and may be a source of reducing power and a substrate for major constituents of cell wall structural proteins in young, metabolically active tissues (Hare and Cress, 1997; Nanjo et al., 1999). In Arabidopsis, the gene encoding Pro dehydrogenase, an enzyme responsible for Pro oxidation and deamination, is not expressed during seed filling but is induced during germination by rehydration and Pro (Nakashima et al., 1998).

Protein hydrolysis along with amino acid oxidative deamination is probably the source of ammonium in the germinating maize kernel because its accumulation has already been observed in germinating seeds of several plant species (Lea and Joy, 1983) including pea and *Medicago truncatula* (G. Glevarec and A. Limami, unpublished data). In Arabidopsis, inhibition of germination triggered by the phytohormone ABA was shown to be associated with a 50% decrease in protein hydrolysis, amino acid catabolism, and ammonium release (Garciarrubio et al., 1997). Ammonium recycling is therefore likely to occur through the reaction catalyzed by the enzyme GS consistent with the increase in Gln content concomitant to the

increase in the enzyme activity. Because NADH-dependent GOGAT activity increases during germination and is largely predominant compared with its Fd-dependent counterpart, it can be therefore hypothesized that the enzyme regenerates Glu for the reaction catalyzed by GS. An increase in the GDH-aminating activity was also observed, which represents a typical response to increased ammonium concentration in plant organs or tissue (Lea and Ireland, 1999). However, ^{15}N-labeling experiments have demonstrated that in 4-d-old maize-germinating seedlings, GDH catalyzes the oxidative deamination of ^{15}N-Glu rather than the amination of 2-oxoglutarate (Stewart et al., 1995). Nevertheless, considering that the latter experiment was carried out on seedlings deprived of their endosperm, which constitutes a non-negligible source of ammonium, it cannot be completely ruled out that part of the ammonium released from protein breakdown and/or deamination of amino acids may be re-assimilated via the GDH-aminating activity. The ratio of aminating to deaminating GDH activity was found to vary depending on the organ or even the physiological status of a given organ (Masclaux et al., 2000; Ferrario-Méry et al., 2002). Because of its involvement in either C or N metabolism, it has been proposed that the shift in the orientation of GDH activity would be an adaptive mechanism to Glu or the C skeleton requirement of the cell (Lea and Ireland, 1999).

Genetic Analysis of N Metabolism in Relation to Maize Kernel Germination

Because the enhancement of Gln synthesis was one of the most important metabolic events occurring during germination, this observation lead to the logical conclusion that the reaction catalyzed by GS may play a major role in the germination process. A quantitative genetic approach was therefore undertaken to support this hypothesis by associating germination trait (T50), kernel size/weight (TKW), a trait linked to germination performance, and kernel GS activity to molecular markers on a genetic map of maize. In germinating maize kernels, three QTLs for GS activity were detected. Two corresponded to GS activity in the early stages of germination (48 h). They colocalized with two of the structural genes for cytosolic GS, *gln3* on chromosome 4 and *gln4* on chromosome 5 (Hirel et al., 2001). This result suggests that for these two genes, the cytosolic enzyme activity in the

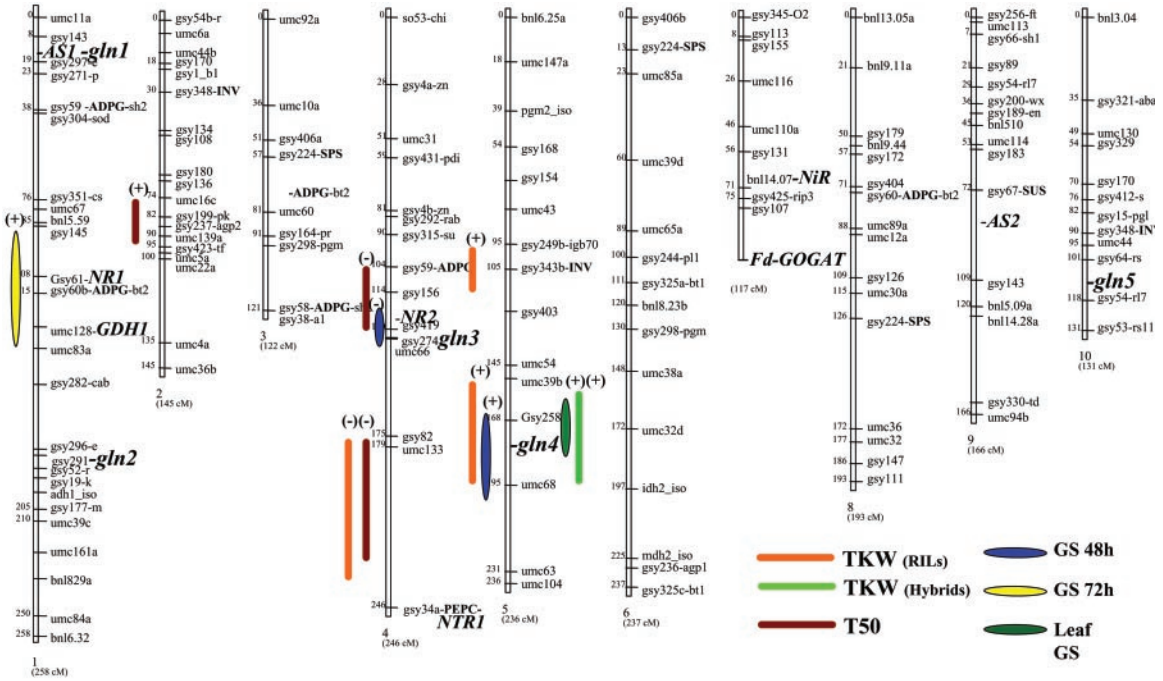

Figure 5. Coincidences between QTLs for GS activity and traits related to grain germination efficiency. Location of the QTLs for GS activity on the maize RFLP genetic map are indicated by oval symbols: blue for grain GS activity after 48 h of germination, yellow for grain GS activity after 72 h of germination, and green for leaf GS activity (see Hirel et al., 2001). Vertical bars indicate location of the QTLs for germination and agronomic traits. Yellow bar, TKW of the RILs; green bar, TKW of the hybrids (see Hirel et al., 2001); red vertical bar, T50. Favorable allele from the parental line Io is indicated by (+) and unfavorable allele by (−). The position of the loci for genes encoding enzymes involved in N assimilation is indicated in bold italics: *AS1* and *AS2*, Asn synthetase 1 and 2; *Fd-GOGAT*, Fd-dependent Glu synthase; *GDH1*, GDH 1; *gnl1* to *5*, GS 1 to 5; *NR*, nitrate reductase; *NiR*, nitrite reductase; and *NTR1*, high-affinity nitrate transporter. The position of the *loci* for genes encoding enzymes involved in C assimilation is indicated in small bold characters (see Causse et al., 1996). ADPG, ADP Glc pyrophosphorylase; INV, invertase; SPS, Suc phosphate synthase.

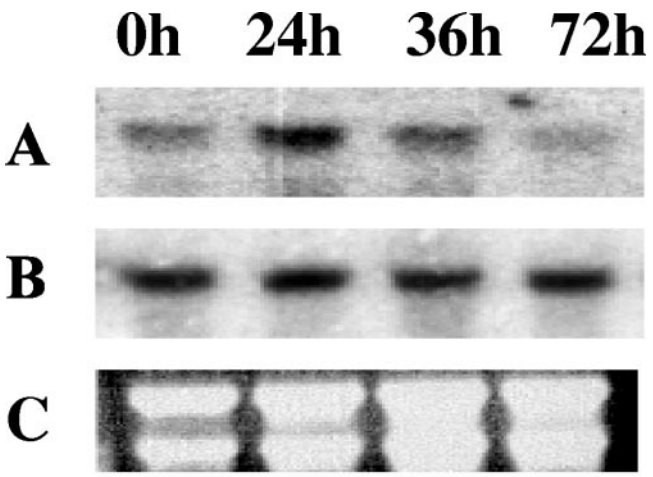

Figure 6. Expression of genes encoding two maize cytosolic GS isoforms *gln3* and *gln4* throughout germination. Northern blots were performed on total RNA extracted at different times following imbibition from kernels of the line Io germinating at 25°C; probes corresponded to specific 5′-untranslated region of Gln1-4 (A) and Gln1-3 (B). C, Ethidium bromide-stained gel showing that similar amounts of total RNAs were loaded in each track.

kernel is regulated at the transcriptional level. The third QTL corresponding to GS activity at later stages of germination (72 h) was detected on chromosome 1. However, no colocalization with any of the two structural genes *gln1* and *gln2* was found, which suggests that other loci located in this region of the chromosome may be involved in controlling the final enzyme activity at this stage of kernel germination. After northern-blot analysis, we found that in the parental line Io, out of the five genes encoding cytosolic GS, only *gln3* and *gln4* were expressed during germination. *Gln3* was transiently expressed, exhibiting a maximum between 24 and 36 h, whereas *gln4* was constitutively expressed during the 72 h of germination. This result is consistent with the detection of two QTLs for GS activity on two different chromosomes each colocalizing with the *gln3* and *gln4* loci. In addition, this finding reinforces the current consensus that each individual GS gene product in a given plant species may play a specific role during plant growth and development (Cren and Hirel, 1999). In maize, it has been previously shown that the five members of the cytosolic GS multigene family may be differentially expressed in different organs including roots and leaves (Sakakibara et al., 1992b; Li et al., 1993) and developing seeds (Rastogi et al., 1998). It has also been demonstrated that, at least in vegetative organs, the source of N may be an additional factor that may modulate their transcription (Sakakibara et al., 1992a). In the present study, we show that two members of the GS1 multigene family (*gln3* and *gln4*) are also expressed in seeds during germination. However, it is still unknown whether the corresponding enzyme activity reflects a distinct or a similar physiological function when present in different organs. Nevertheless, the identification of a QTL for GS activity colocalizing with both the *gln3* structural gene and a QTL for T50 on chromosome 4 (both with the favorable allele from the parental line, Io) suggests that its transient expression may be of major importance in the control of germination efficiency. This hypothesis is further supported by the highly significant negative correlation between GS activity and T50 ($P < 0.001$ and $r = -0.30$). This means that kernels exhibiting a faster germination rate (low T50) have a higher GS activity.

On chromosome 5, the triple colocalization between *gln4*, another structural gene encoding GS1, and two QTLs for GS activity and TKW is also an interesting finding that may help to unravel the physiological function of the corresponding translation product. In a previous study, a colocalization between leaf GS activity in young developing maize plants and the same GS1 structural gene was identified (Hirel et al., 2001). Moreover, in this region of chromosome 5, a QTL for TKW reflecting the agronomic performances of the hybrids derived from the RILs population was identified in the same position (Fig. 5). This result shows that the RILs and derived hybrids share common QTLs for TKW both with the favorable allele from the parental line Io.

Both of these observations confirm that *gln4* is a useful candidate gene that may influence grain yield and as a consequence grain size, of which the latter may be one of the main components controlling the efficiency of germination. *Gln4* is constitutively expressed both during seed germination and in vegetative tissues (Sakakibara et al., 1992a), regardless of the developmental stage of the plant (B. Hirel and E. Carayol, unpublished data). This result further supports the hypothesis that *gln4* is a housekeeping gene (Hirel et al., 2001).

Genetic Analysis of Maize Germination in Relation to Kernel Size

The observation in the parental lines Io and F_2 that smaller kernels resulted in higher germination efficiency suggests that kernel size is an important factor influencing the germination efficiency. The hypothesis that the association of both traits is genetically controlled is also supported by the fact that differences in the germination efficiency of Io and F_2 did not rely on differences in the rehydration speed. Moreover, on chromosome 4, we found a coincidence between two QTLs related to the germination efficiency (T50 and TKW), both with the favorable allele from the parental line F2. This observation suggests that both traits are controlled by the same gene(s) or by tightly clustered genes in this chromosomal region. Statistical data further support this hypothesis because both traits show a highly significant ($P < 0.001$) positive correlation ($r = 0.29$). A putative candidate gene colocalizing with these two QTLs was

found in maize genetic maps (University of Missouri, Columbia; http://www.agron.missouri.edu/Coop/binsmaps.html). This gene named knotted related homeobox7, which belongs to class 2 kn1-like homeobox genes (Kerstetter et al., 1994; Bharathan et al., 1999), was shown in Arabidopsis to be expressed only during early organ development particularly near the junction between roots and hypocotyls in young seedlings (Serikawa et al., 1996, 1997). Further studies will be required to monitor the expression of knotted related homeobox7 during maize kernel germination and to validate function in transgenic plants or mutants.

CONCLUSIONS

Study of inheritance of quantitative traits has become more powerful with the advent of molecular markers that allowed for construction of genetic maps and detection of QTLs for complex traits such as crop yield and their components. QTL mapping was also shown more recently to be a very powerful tool for the analysis of complex physiological traits such as N use efficiency (Prioul et al., 1997; Agrama et al., 1999; Hirel et al., 2001; Limami and de Vienne, 2001). This type of approach undertaken in model species such as maize (Hirel et al., 2001), rice (Obara et al., 2001), and Arabidopsis (Rauh et al., 2002) resulted in the identification of putative candidate genes encoding enzymes involved in either N assimilation or recycling that may exert a major control on either plant productivity or plant growth. Among these candidate genes, those encoding GS were found to be always associated with agronomic or developmental traits, outlining the central role of the enzyme in the control of plant growth and development (Limami et al., 1999; Miflin and Habash, 2002). In addition, in this study, we have shown by combining a physiological and a quantitative genetics approach that at least one member of the maize cytosolic GS multigene family (*gln4* locus located on chromosome 5) is likely to play an ubiquitous role throughout plant development, from seed germination to seed filling. In contrast, other members of the multigene family such as *gln3* located on chromosome 3 may be more specifically involved in the control of other developmental processes including the efficiency of germination.

MATERIALS AND METHODS

Plant Material for Germination Studies

A total of 140 RILs and the parental lines a French flint and early line of maize (*Zea mays*; F_2) and an iodent late line (Io) were germinated in petri dishes (diameter 9 cm) on paper (Whatman, Clifton, NJ) soaked with deionized water and maintained in a growth chamber in darkness at either 21°C or 25°C.

Three replicates of 40 grains per petri dish were used for germination test (T50). Germination time was determined as the time of radicle emergence. Comparison of germination rate and efficiency between the various lines was based on the determination of the T50, i.e. the time at which 50% of grains germinated. Thirty grains per line were weighed; the result was expressed as TKW and was used to characterize either kernel size or weight.

At various stages of the germination process, grains of each replicate were collected, pooled, and frozen in liquid N_2. Part of this sample was ground and stored as a powder at −80°C for further biochemical analysis. Another part of this sample was lyophilized, ground, and stored as a powder at −80°C for metabolite analyses.

GS and GDH activity were determined in the 140 RILs and the parental lines Io and F2. Metabolite analyses (total amino acids and ammonium), determination of individual amino acids by ion-exchange chromatography, enzymes activities (GS, NADH-GDH, NAD-GDH, and PEPc), and northern analysis for expression of GS genes (*gln1–gln5*) were carried out on parental lines Io and F2.

The RILs were F_6 generation derived from a cross between F_2 and Io, and such a population of RILs was chosen because the two parental lines are highly complementary in terms of heterotic grain productivity. Traits used for correlation studies and QTL detection were the germination trait (T50), the grain size/weight trait (TKW), and the physiological trait GS activity.

Protein Extraction, Enzymatic Assay, Metabolite Extraction, and Analyses

Protein extraction was carried out on 250 mg of frozen material as described previously (McNally et al., 1983). GS activity was assayed using the biosynthetic activity as described by O'Neal and Joy (1973). GS activity was expressed as nanomoles per minute per milligram of protein. Glu dehydrogenase NAD(H)-GDH were measured as described by Turano et al. (1996), except that extraction buffer was the same as for GS. NAD(H)-GDH activity was expressed as nanomoles of Glu used (or produced) per minute per milligram of protein. Fd-GOGAT activity was measured as described by Suzuki et al. (1994) using methyl viologen as electron donor. NADH-GOGAT was measured according to the same method, however, with NADH as electron donor instead of methyl viologen. Either Fd-GOGAT or NADH-GOGAT activities were expressed as nanomoles of Glu produced per minute per milligram of protein. PEPc activity was measured by the coupling of this activity to malate dehydrogenase, which transforms oxaloacetate produced by PEPc into malate using NADH as reducing power. The activity was expressed as nanomoles of NADH consumed per minute per milligram of protein.

Proteins were quantified using the Bradford (1976) method. About 20 mg of lyophilized material were extracted in 1 mL of 80% (v/v) ethanol for 1 h. During extraction, the samples were continuously agitated and then spun for 5 min at 15,000 rpm. The supernatant was recuperated, and the pellet was subjected to further extractions in 60% (v/v) ethanol and finally in water. All supernatants were combined to form the hydroalcoholic extract.

Ammonium was determined by the phenol hypochlorite assay (Berthelot reaction). Total free amino acids were determined by the Rosen (1957) colorimetric method with Leu as a reference. Individual amino acid composition was determined by ion-exchange chromatography (Rochat and Boutin, 1989).

RNA Extraction and Northern Blotting

Total RNA was extracted as described by Verwoerd et al. (1989) from the frozen grain material. Northern-blot analysis was performed as described previously (Ferrario-Méry et al., 1998). The probes used for mRNA detection corresponded to 5′-untranslated region sequences specific to each GS isoform (Sakakibara et al., 1992b).

Gene Mapping and Detection of QTLs

For the mapping of genes encoding enzymes and proteins involved in N metabolism, we used the RFLP genetic map published by Causse et al. (1996) containing 152 marker loci corresponding to a total map length of 1,813 cM. The mean interval between two markers, depending on the chromosome, varies from 8 to 18 cM. Mapping of the genes encoding proteins and enzymes involved in N metabolism was performed as described previously (Hirel et al., 2001).

QTLs were detected using the QTL software (Utz and Melchinger, 1995) following simple interval mapping. Linkage between a given QTL and a marker on genetic map was determined by the logarithm of the odds (LOD)

score approach (Hui Lui, 1997). Only QTLs with a LOD score greater than 2 were considered (Lander and Botstein, 1989). To represent a QTL on the map taking into account error in the location, we gave the chromosome region corresponding to a LOD greater than the maximum LOD minus 1, which is not a true confidence interval; it is called a LOD-1 interval. It generally overestimates the confidence interval. Two QTLs of different traits will be declared as coincident when their LOD-1 intervals overlap. A coincidence will be said to be positive when there is coincidence of favorable (or unfavorable) alleles for both traits. The coincidence will be said to be negative when there is coincidence of a favorable allele for one trait with an unfavorable allele for the other trait. For each QTL detected, the estimated additive effect (one-half of the difference between the estimated value of the two homozygous genotypes at the QTL) is presented.

Statistical Analysis

To determine whether differences in germination trait (T50) between the lines are attributable to genotypic characteristics or germination environment, the results were subjected to variance analysis (Fisher test). Statistic software JMP (University of Knoxville, TN) was used for calculating correlations between characters.

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