

The *lys5* Mutations of Barley Reveal the Nature and Importance of Plastidial ADP-Glc Transporters for Starch Synthesis in Cereal Endosperm¹

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Much of the ADP-Glc required for starch synthesis in the plastids of cereal endosperm is synthesized in the cytosol and transported across the plastid envelope. To provide information on the nature and role of the plastidial ADP-Glc transporter in barley (*Hordeum vulgare*), we screened a collection of low-starch mutants for lines with abnormally high levels of ADP-Glc in the developing endosperm. Three independent mutants were discovered, all of which carried mutations at the *lys5* locus. Plastids isolated from the *lys5* mutants were able to synthesize starch at normal rates from Glc-1-P but not from ADP-Glc, suggesting a specific lesion in the transport of ADP-Glc across the plastid envelope. The major plastidial envelope protein was purified, and its sequence showed it to be homologous to the maize (*Zea mays*) ADP-Glc transporter BRITTLE1. The gene encoding this protein in barley, *Hv.Nst1*, was cloned, sequenced, and mapped. Like *lys5*, *Hv.Nst1* lies on chromosome 6(6H), and all three of the *lys5* alleles that were examined were shown to carry lesions in *Hv.Nst1*. Two of the identified mutations in *Hv.Nst1* lead to amino acid substitutions in a domain that is conserved in all members of the family of carrier proteins to which Hv.NST1 belongs. This strongly suggests that *Hv.Nst1* lies at the *Lys5* locus and encodes a plastidial ADP-Glc transporter. The low-starch phenotype of the *lys5* mutants shows that the ADP-Glc transporter is required for normal rates of starch synthesis. This work on Hv.NST1, together with the earlier work on BRITTLE1, suggests that homologous transporters are probably present in the endosperm of all cereals.

Starch synthesis in the plastids of all plants requires the nucleotide sugar ADP-Glc as a Glc donor in the reaction catalyzed by starch synthase. ADP-Glc is synthesized by ADP-Glc pyrophosphorylase (AGPase) from Glc-1-P and ATP. In most plant cells, this reaction occurs exclusively in the plastids. However, in the endosperm of graminaceous plants, the major form of AGPase is extraplastidial (Denyer et al., 1996; Thorbjørnsen et al., 1996; Beckles et al., 2001; Sikka et al., 2001; Tetlow et al., 2003). Mutants of cereal species that lack the cytosolic form of AGPase, such as the shrunken2 and brittle2 mutants of maize (*Zea mays*; Giroux and Hannah, 1994) and the barley (*Hordeum vulgare*) mutant Risø 16 (Johnson et al., 2003), make less starch than normal, showing that synthesis of ADP-Glc in the cytosol is necessary for normal rates of starch synthesis in the endosperm.

Graminaceous endosperm requires an ADP-Glc transporter in the plastid envelope to import the ADP-Glc synthesized in the cytosol to the site of starch synthesis in the plastid stroma. The identity of this nucleotide-sugar transporter (NST) is known for maize. The *Brittle1* (*Bt1*) locus encodes a plastidial NST located in the inner membrane of the plastid envelope that is capable of transporting ADP-Glc (Li et al., 1992; Shannon et al., 1998) in exchange for AMP or ADP (Möhlmann et al., 1997). It is clear that BT1 is the major transporter of ADP-Glc in the plastid envelopes of maize. In its absence, ADP-Glc synthesized by the cytosolic isoform of AGPase accumulates in the cytosol and the rate of starch synthesis is 80% lower than normal (Tobias et al., 1992).

The nature of the ADP-Glc transporter in the plastids of other cereal endosperms is not clear. First, there are *Bt1*-like genes in cereals other than maize, but *Bt1*-like genes also occur in non-cereals such as Arabidopsis (e.g. At4g32400). This means that BT1-like proteins probably have different physiological roles in different species. Even the *Bt1*-like genes in various cereal species may have different roles. Second, an antiserum raised to maize BT1 failed to cross-react with any protein in starchy tissues from species other than maize, including other cereal grains (Cao and Shannon, 1997). This raised the possibility that the ADP-Glc transporter in species other than maize might be unrelated to the BT1 protein. Third, an ADP-Glc transporter was identified in the plastid envelopes of wheat endosperm (Emes et al., 2001). Preliminary

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attempts to purify this also supported the idea that ADP-Glc transporters unlike BT1 existed in other cereal species (Tetlow et al., 1999).

The aim of this study was to discover the nature and importance of the ADP-Glc transporter in barley endosperm by studying mutants that are unable to transport ADP-Glc into the plastid. To identify such mutants, we measured the ADP-Glc contents of the developing endosperms of a collection of barley mutant lines with low-starch content in the grains and compared these values to those of wild-type barley endosperms. We postulated that mutants with reduced capacity to transport ADP-Glc across the plastid envelope would have elevated levels of ADP-Glc in the endosperm. This is the case in the *Bt1* mutants of maize. The ADP-Glc content in the endosperms of *Bt1* mutants is 11.5-fold higher than that in the wild type (Shannon et al., 1996). This approach allowed us to identify the barley ADP-Glc transporter Hv.NST1, a homolog of maize BT1, which is required for normal rates of starch synthesis in the endosperm.

RESULTS AND DISCUSSION

Identification of ADP-Glc-Accumulating Mutants

The ADP-Glc, UDP-Glc, and starch contents of the developing endosperm of a collection of low-starch mutants representing a number of different complementation groups were measured and compared with those of two wild-type barley lines, Bomi and Carlsberg II (Table I). One line, Risø 13, had an ADP-Glc content that was more than 7-fold higher than normal.

Risø 13 belongs to a complementation group consisting of four independently isolated allelic mutants (Table II). Three of these mutant lines, Risø 13, Risø 29, and Risø 86, were originally selected from ethyl

methyl sulfonate (EMS)-mutagenized populations of Bomi and Carlsberg II as high-Lys mutants (Doll, 1983), and the mutated locus was designated *lys5* (Jensen and Doll, 1979). Like most of the other high-Lys mutants of barley, they were subsequently found to have shrunken endosperm and a low seed weight, and Risø 13 was also shown to have a low-starch content (Table II). We have reported previously that two other high-Lys barley mutants, Risø 16 (Johnson et al., 2003) and Risø 17 (Burton et al., 2002a), have lesions in enzymes involved in starch biosynthesis (AGPase and isoamylase, respectively). Thus, the effect on Lys content is probably a secondary effect of a lesion in the pathway of starch biosynthesis. The Risø *lys5* mutants were found to be allelic to a previously identified spontaneous mutation at a locus named variously *se6*, *sex1a*, and *sex1f* (Jensen, 1979). It was later proposed that this locus be renamed *lys5e* (Jensen and Doll, 1979).

In an experiment separate from that shown in Table I, measurements of the ADP-Glc and UDP-Glc contents of two of the other *lys5* mutants, Risø 29 and Risø 86, were compared with those of Risø 13 and the wild-type, parental varieties Bomi and Carlsberg II (Fig. 1). The UDP-Glc contents of the *lys5* mutants were not statistically significantly different from those of the wild types in either experiment (Table I; Fig. 1). The value for the ADP-Glc content for Risø 13 in the second experiment (Fig. 1) differed from the value obtained for the batch of plants grown previously (Table I) but was again significantly higher than those of the wild types grown and harvested at the same time. The ADP-Glc contents of Risø 29 and Risø 86 were also higher than those of the wild types. This suggests that high ADP-Glc content is a phenotype determined by mutations at the *Lys5* locus.

As expected, the starch contents of the mutants were lower than those of the wild types (Table I). The reduction in starch content in Risø 13 (62% of normal)

Table I. Metabolite contents of wild-type and mutant endosperm

Developing endosperm of 12 to 37 mg fresh weight was extracted as described in "Materials and Methods" and assayed for ADP-Glc and UDP-Glc. Values are means \pm SD of measurements made on a minimum of four independent extracts. Mature endosperm was extracted as described in "Materials and Methods" and assayed for starch. Values are means \pm SD of measurements made on a minimum of three independent extracts. All plants were grown at the same time under identical conditions.

Cultivar/Line	ADP-Glc <i>nmol g⁻¹ fresh weight</i>	UDP-Glc <i>nmol g⁻¹ fresh weight</i>	Starch <i>mg per endosperm</i>
Wild types			
Bomi	184.5 \pm 43.0	289.8 \pm 53.1	19.7 \pm 2.9
Carlsberg II	174.5 \pm 24.1	296.7 \pm 68.0	19.8 \pm 1.2
Low-starch mutants			
Risø 13	1418.0 \pm 387.6	443.05 \pm 236.1	12.2 \pm 0.3
Risø 16	84.5 \pm 46.1	609.9 \pm 93.8	11.7 \pm 2.9
Risø 17	236.1 \pm 30.6	227.5 \pm 30.0	10.2 \pm 1.4
Risø 527	175.7 \pm 36.4	297.3 \pm 60.9	14.0 \pm 1.7
Risø 1508	175.5 \pm 51.2	205.31 \pm 46.8	17.2 \pm 3.2
Notch 1	131.6 \pm 50.1	278.8 \pm 82.2	17.3 \pm 1.7
Notch 2	217.0 \pm 55.8	413.9 \pm 38.3	4.8 \pm 2.9

Table II. Barley mutants carrying lesions at the *Lys5* locus

References are indicated by footnotes.

Line	Parental Cultivar	Locus	Mutagen	Seed Weight
Risø 13	Bomi	<i>lys5f</i>	EMS	% Parental 74 ^a , 80 ^b
Risø 29	Carlsberg II	<i>lys5g</i>	EMS	88 ^b ,
Risø 86	Carlsberg II	<i>lys5h</i>	EMS	79 ^b
<i>se6, sex1a, sex1f</i>	Compana	<i>lys5e</i>	Spontaneous	81 ^c , 75 ^d

^aThis study. ^bDoll (1976). ^cUllrich and Eslick (1978).
^dJarvi and Eslick (1975).

was comparable to that in Risø 16 (59% of normal), a mutant in the same genetic background (Bomi) that lacks cytosolic AGPase activity (Johnson et al., 2003). Mutations that prevent the transport of ADP-Glc into the plastid and ones that prevent the synthesis of ADP-Glc in the cytosol would be expected to affect starch synthesis to a similar extent. This result is therefore consistent with the hypothesis that the *lys5* mutants may be unable to transport ADP-Glc across the plastid envelope.

Starch Granule Morphology

The morphology of the starch granules is severely affected in Risø 13 (Fig. 2). They are smaller than normal and abnormally shaped, each having a conspicuous peripheral groove and sunken cheeks. Similar changes in granule morphology were noted for Risø 16 (Johnson et al., 2003). This shows that the morphology of the mutant granules is not specific to either genetic lesion and may be due to the similar reductions in starch accumulation in these mutants.

Plastids Isolated from the Endosperm of Risø 13 Have a Reduced Capacity to Synthesize Starch from ADP-Glc

To discover whether the transport of ADP-Glc into endosperm plastids is affected in *lys5* mutants, we isolated plastids from the developing endosperm of wild-type barley (Bomi) and one of the *lys5* mutants, Risø 13, and studied their ability to take up metabolites and make starch (Table III). Control incubations, in which the plastids were deliberately ruptured, were performed for each plastid preparation to allow correction for the rates of synthesis of starch by free starch granules. Intact plastids from the wild type were able to synthesize starch from ADP-Glc at rates (36 nmol Glc min⁻¹ per unit of alkaline pyrophosphatase activity; Table III) similar to the estimated in vivo rate of starch synthesis (approximately 70 nmol Glc min⁻¹ per unit of alkaline pyrophosphatase activity; estimated for endosperm of 10 mg fresh weight from values in Johnson et al., 2003). Wild-type plastids could also synthesize starch from Glc-1-P, but they did so at rates

less than one-tenth of those with ADP-Glc. Plastids from the mutant were able to take up Glc-1-P and convert this to starch as readily as plastids isolated from wild-type endosperm. However, the rate of starch synthesis from ADP-Glc was less than 30% of that for the wild-type plastids. This suggests that Risø 13 has a reduced capacity to import ADP-Glc into endosperm plastids.

ADP-Glc Does Not Accumulate in *lys5* Mutants in the Absence of Cytosolic AGPase

The results above are consistent with the idea that the *lys5* mutations cause a specific defect in ADP-Glc transport into endosperm plastids. If this is true, then the mutation in Risø 16, which eliminates the activity of the cytosolic AGPase, should be epistatic to mutations at the *Lys5* locus, and the double mutant plants should not accumulate ADP-Glc to abnormally high levels. To test this, we made double mutants by crossing Risø 13 and Risø 16. Two double-mutant lines were selected from the progeny of this cross (see "Materials and Methods"; data not shown). The double mutants were grown together with three of the single *lys5* mutants and two wild-type parental lines, and their ADP-Glc contents and grain weights were measured.

The ADP-Glc content of the double mutants was much lower than that of the single *lys5* mutants and similar to that of the wild types (Fig. 1). This suggests that the Risø 16 mutation is epistatic with respect to the *lys5* mutation in Risø 13, and this is consistent with the *lys5* mutation affecting a step in the starch biosynthetic pathway subsequent to cytosolic ADP-Glc

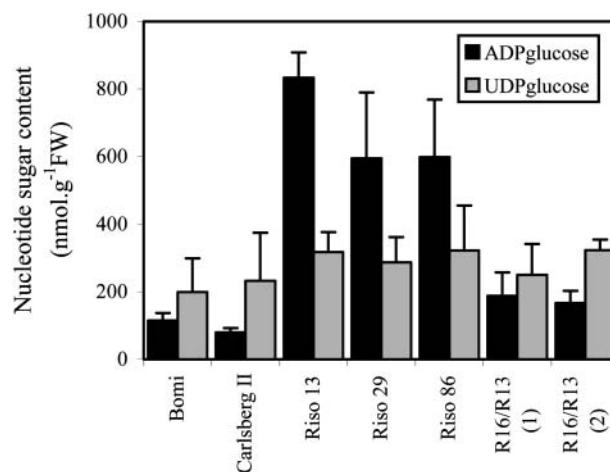


Figure 1. Metabolite contents of wild-type and mutant endosperm. Developing endosperm of 12 to 37 mg fresh weight was extracted as described in "Materials and Methods" and assayed for ADP-Glc and UDP-Glc. Values are means \pm SD of measurements made on a minimum of four independent extracts each for endosperm from separate plants. The metabolite contents of endosperm from two double mutant plants [R16/R13 (1) and (2)] containing the low-starch mutations from both Risø 16 and Risø 13 (*lys5*) were also determined. All plants were grown at the same time under identical conditions.

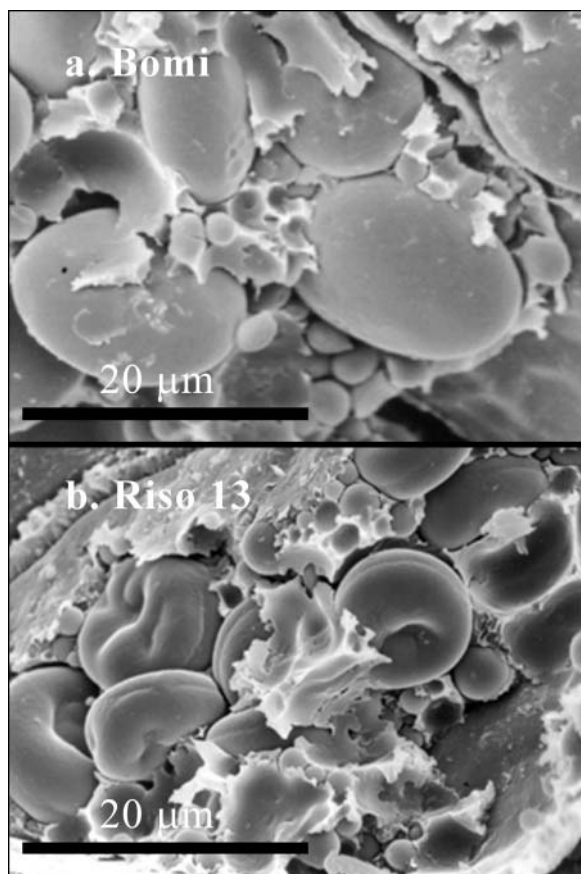


Figure 2. Scanning electron microscopy of barley grains. Mature grains were fractured to reveal the starch granules within the endosperm cells and viewed in a scanning electron microscope. The magnifications are indicated by the scale bars.

synthesis—such as ADP-Glc transport. However, for plants grown at the same time under identical conditions, the average grain weights of the two double mutant lines (means \pm SD for 3–5 separate plants: 38.9 ± 1.3 mg and 35.7 ± 2.8 mg) were lower than those of either of the single mutant parents (means \pm SD for 4–5 separate plants: Risø 16, 45.6 ± 2.1 mg; Risø 13, 42.7 ± 4.3 mg). Thus, there is an interaction between the Risø 13 and Risø 16 mutations with respect to grain weight that cannot be explained at present.

The *lys5* Mutants Have Normal Activities of Starch Biosynthetic Enzymes in the Endosperm

The maximum catalytic activities of enzymes involved in the conversion of Suc to starch in the developing endosperm of the *lys5* mutants were compared with those in the respective parental cultivars, Bomi and Carlsberg II (Table IV). This showed that there were no consistent differences in enzyme activities between the mutants and the wild types. For example, there were no statistically significant differences between Risø 29 and Bomi in any of the enzyme

activities measured. In particular, no reductions in enzyme activity were observed that could potentially contribute to the reduced rate of starch synthesis in the *lys5* mutants.

The Major Protein in the Plastid Envelope Is the ADP-Glc Transporter

To discover whether the plastid envelopes in barley endosperm contain a protein similar to the BT1 protein that is responsible for the transport of ADP-Glc in maize, we isolated plastid envelope proteins from wild-type endosperm and subjected them to SDS-PAGE (Fig. 3). The most abundant protein had a molecular mass of approximately 41 kD. Direct sequencing of the N terminus of this protein and sequencing of internal tryptic fragments using quadrupole-time-of-flight (Q-TOF) mass spectrometry (MS) showed that it was similar to the maize BT1 protein (Fig. 4). Plastid envelope proteins were also isolated from the mutants Risø 13, Risø 29, Risø 86, and the wild-type Carlsberg II and analyzed on SDS-polyacrylamide gels. This comparison revealed no differences between the mutants and their parental wild types in the abundance or size of the BT1-like protein (Fig. 4; data not shown).

An antiserum raised to the C-terminal 56 amino acids of the maize BT1 protein (kind gift of Tom Sullivan, University of Wisconsin, Madison) failed to recognize the barley BT1-like protein in immunoblots of the barley plastid envelope proteins (data not shown). Cao and Shannon (1997) also reported that the same antiserum failed to react with proteins of

Table III. Starch synthesis by isolated plastids

Plastids were isolated from developing wild-type (Bomi) or mutant (Risø 13) endosperm and incubated for 30 min at 25°C in the presence of 3 mM labeled ADP-Glc or Glc-1-P and 0.5 mM ATP. For each plastid preparation, the rates of starch synthesis were measured in triplicate samples containing intact plastids and triplicate samples containing deliberately ruptured plastids. The rates of starch synthesis that were dependent on plastid intactness were calculated. To allow comparison between plastid preparations, the ADP-Glc- and Glc-1-P-dependent rates of starch synthesis are expressed per unit ($\mu\text{mol min}^{-1}$) of alkaline pyrophosphatase activity. Values are means \pm SE for five independent plastid preparations. Comparison of the incorporation of Glc into starch for plastids isolated from Risø 13 and Bomi was done using Microsoft Excel software (*t* test, two-tailed distribution, two-sample equal variance). This statistical analysis showed that for ADP-Glc, the difference between Bomi and Risø 13 was statistically significant (Student's *t* test, $P < 0.01$), but for Glc-1-P, the rates were not statistically significantly different ($P > 0.10$).

Cultivar/Line	Incorporation of Glc into Starch	
	ADP-Glc	Glc-1-P
	<i>nmol min⁻¹ per unit alkaline pyrophosphatase activity</i>	
Bomi	36.02 ± 3.47	2.96 ± 0.44
Risø 13	10.48 ± 2.39	4.04 ± 0.87

Table IV. Comparison of the maximum catalytic activities of enzymes in crude extracts of developing endosperm

Developing endosperm from grains of 45 to 55 mg fresh weight was extracted as described in "Materials and Methods" and assayed for enzyme activities. The activity of starch-branching enzyme could not be determined in these extracts due to the presence of interfering activities. All plants were grown at the same time under identical conditions. Values are means \pm SE of measurements made on the number (shown in brackets) of independent extracts of grains from separate plants. Comparison of the activities of each enzyme in the *lys5* mutant line and its respective wild-type cultivar (see Table II) was done using Microsoft Excel software (*t* test, two-tailed distribution, two-sample equal variance). This statistical analysis showed that the activities of fructokinase, glucokinase, ADP-Glc pyrophosphorylase, soluble starch synthase, and Suc synthase were higher in Risø 13 than in Bomi ($P < 0.01$), and the activity of soluble starch synthase was higher in Risø 86 than in Carlsberg II ($P < 0.01$). The activity of phospho-Glc isomerase in Risø 86 was lower than that in Carlsberg II, but the statistical significance of this change was marginal ($P = 0.04$). All other pair-wise comparisons showed no statistically significant difference ($P > 0.08$).

Enzyme	Activity				
	Wild Types		Low-Starch Mutants		
	Bomi	Carlsberg II	Risø 13	Risø 29	Risø 86
	$\mu\text{mol min}^{-1} \text{g}^{-1}$ fresh weight				
Suc synthase	3.44 \pm 0.17 [5]	3.73 \pm 0.32 [4]	5.09 \pm 0.12 [5]	5.30 \pm 0.76 [5]	4.09 \pm 0.08 [3]
UDP-Glc pyrophosphorylase	54.88 \pm 8.56 [4]	64.53 \pm 6.30 [4]	78.41 \pm 7.70 [5]	65.72 \pm 9.01 [4]	67.78 \pm 10.40 [3]
Fructokinase	0.21 \pm 0.02 [5]	0.20 \pm 0.01 [4]	0.38 \pm 0.04 [5]	0.24 \pm 0.02 [4]	0.19 \pm 0.01 [3]
Glucokinase	0.35 \pm 0.04 [5]	0.45 \pm 0.01 [4]	0.57 \pm 0.03 [5]	0.46 \pm 0.06 [4]	0.38 \pm 0.03 [3]
Phosphoglucomutase	21.05 \pm 5.09 [5]	44.75 \pm 6.37 [4]	27.45 \pm 5.15 [4]	39.65 \pm 9.71 [4]	39.25 \pm 10.23 [3]
Phospho-Glc isomerase	17.38 \pm 2.99 [5]	15.68 \pm 0.64 [4]	23.86 \pm 3.99 [5]	22.44 \pm 4.31 [4]	13.53 \pm 0.23 [3]
ADP-Glc pyrophosphorylase	4.97 \pm 0.34 [5]	5.91 \pm 0.85 [4]	9.56 \pm 0.90 [4]	9.65 \pm 2.17 [4]	7.79 \pm 2.23 [3]
Soluble starch synthase	0.18 \pm 0.02 [5]	0.18 \pm 0.05 [3]	0.62 \pm 0.04 [5]	0.38 \pm 0.13 [5]	0.50 \pm 0.03 [3]
Granule-bound starch synthase	0.45 \pm 0.06 [5]	0.49 \pm 0.17 [4]	0.70 \pm 0.11 [5]	0.36 \pm 0.02 [5]	0.55 \pm 0.05 [3]
Alkaline pyrophosphatase	7.69 \pm 1.46 [5]	6.03 \pm 1.80 [4]	6.19 \pm 0.63 [5]	4.96 \pm 0.38 [6]	5.89 \pm 0.36 [3]

similar size to maize BT1 in microsomal membrane preparations from starchy tissues of species other than maize, although they did show a weak cross-reaction to a protein in barley grains that was larger in mass and less abundant than BT1. We did not detect this

protein in our plastid envelope preparations. The reason for the species specificity of the antiserum, given the similarity in sequence of maize BT1 and the barley homolog (see below), is not known.

The Gene Encoding the Barley BT1-Like Protein *Hv.Nst1*

The gene encoding the barley BT1-like protein was cloned and sequenced from barley (cv Bomi) and named *Hv.Nst1* (*Hordeum vulgare* nucleotide sugar transporter 1; GenBank accession no. AY560327). The predicted protein is 71.5% identical and 75% similar to the BT1 protein from maize (Fig. 4). *Hv.Nst1* is identical in sequence, except at the extreme 5' end, to a barley partial cDNA identified previously (GenBank accession no. AAK55487). *Hv.Nst1* has a predicted plastid transit peptide of 56 amino acids, which we have confirmed by N-terminal sequencing of the mature protein purified from barley endosperm plastid envelopes (Fig. 4).

The Chromosomal Location of *Hv.Nst1*

To discover whether the *Hv.Nst1* gene could lie at the *Lys5* locus, we determined the chromosomal location of *Hv.Nst1* using barley-wheat telosomic addition lines (Islam et al., 1981; Islam, 1983). The *Lys5* locus is located on the long arm of chromosome 6, close to the centromere (Jarvi and Eslick, 1975; Jensen, 1979). The *Hv.Nst1* gene is also located on the long arm of chromosome 6 (data not shown). This is consistent with the hypothesis that lesions in *Hv.Nst1* may be responsible for the *lys5* phenotype.

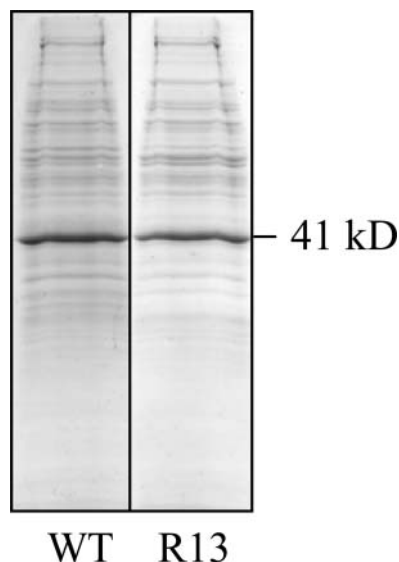


Figure 3. Plastid envelope proteins separated by SDS-PAGE. Plastids were isolated from developing wild-type (WT; Bomi) or mutant (R13; Risø 13) endosperm. After rupture of the plastids, the envelope fraction was isolated by high-speed centrifugation. Envelope proteins were extracted with SDS, separated on a 12.5% SDS-polyacrylamide gel, and stained with Coomassie BioSafe (Bio-Rad, Hercules, CA). The major protein, with an approximate molecular mass of 41 kD, is indicated.

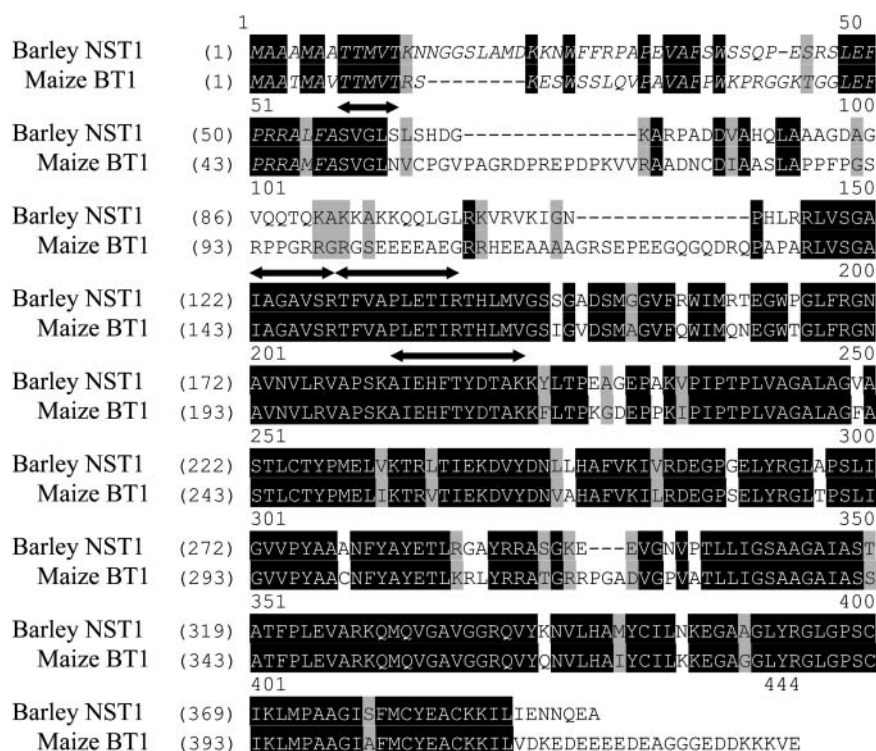


Figure 4. Comparison of the primary sequences of maize BT1 and barley NST1. The primary sequences predicted for maize BT1 and barley NST1 were compared using Vector NTI (InforMax; Invitrogen). Black shading indicates identical amino acids, and gray shading indicates similar amino acids. The arrows show predicted sequence of barley NST1 that matches sequence obtained by direct N-terminal sequencing of the purified protein (SVGLS) or sequencing of tryptic fragments using Q-TOF MS ([L/I]VSGA[L/I]AGAVSR; TFVAP[L/I]GT[L/I]R; A[L/I]EH[F?]-TYDTAK). The transit peptide sequence, predicted from the N-terminal sequence of the barley NST1, is shown in italics.

The *lys5* Mutants Carry Lesions in *Hv.Nst1*

The sequence of *Hv.Nst1* from Bomi was compared with that from a second wild-type cultivar, Carlsberg II, and with the sequences of *Hv.Nst1* from three of the four *lys5* mutants. A single nucleotide polymorphism (C or G at position 548) was found between the two wild-type genes that resulted in a difference between Bomi and Carlsberg II in the amino acid residue at position 184 (Bomi, Ala; Carlsberg II, Gly; Fig. 5b). *Hv.Nst1* in mutant Risø 29, which was derived from Carlsberg II, shares the parental sequence at nucleotide 548 (corresponding to amino acid 184). However, *Hv.Nst1* in mutant Risø 86, which was also derived from Carlsberg II, has the Bomi-type sequence at this position. The reason for this is not clear. It is possible that neither Bomi nor Carlsberg II, nor any of their offspring, are pure breeding for this polymorphism.

All three of the *lys5* mutants also have point mutations in the *Hv.Nst1* gene that lead to other alterations in amino acids. Two of the mutants, Risø 29 and 86, have the same point mutation (T instead of C at position 682) that results in the substitution of a Ser for the Pro residue at position 228. Despite possessing the same nucleotide substitution at position 682, it is likely that these mutations originated individually since the two genes differ at nucleotide position 548 (see above). Mutant Risø 13 has a point mutation (G instead of T at position 818) that results in the substitution of Glu for Val at position 273.

The presence of mutations in the *Hv.Nst1* genes of all three of the independently isolated *lys5* mutants

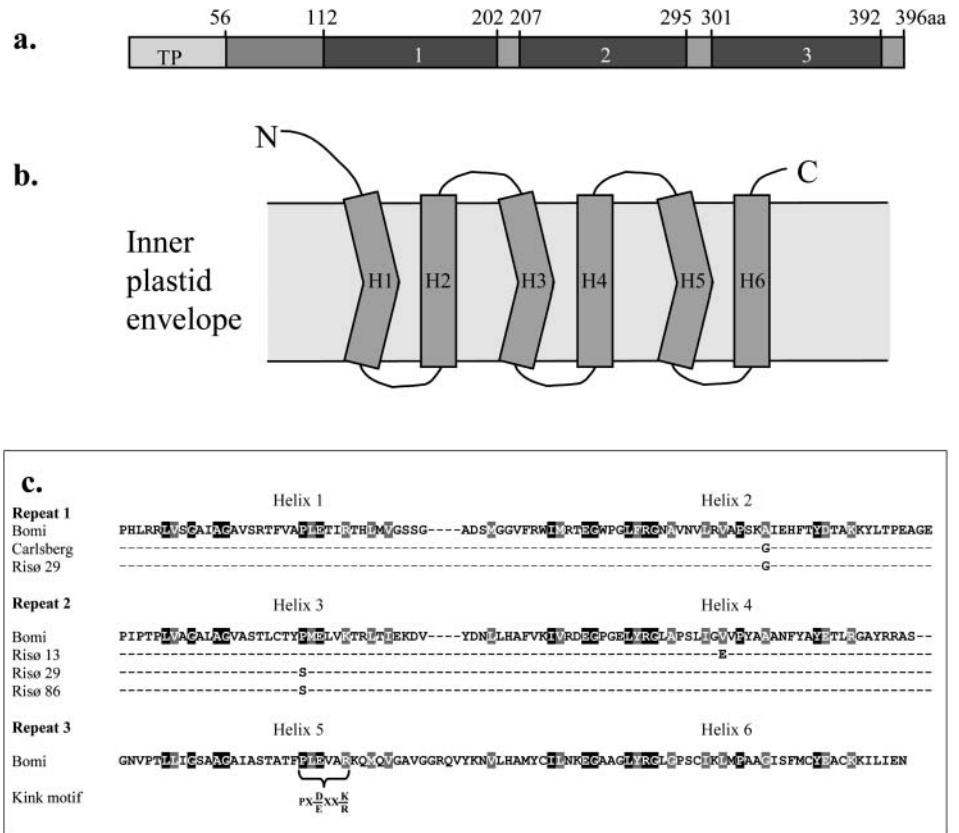
studied makes it highly likely that the *Hv.Nst1* gene lies at the *Lys5* locus. The *Hv.NST1* protein is present in the plastid envelopes of developing endosperms, and the phenotype of the *lys5* mutants suggests that lesions in this gene prevent the transport of ADP-Glc across the plastid envelope. Together, these data suggest that *Hv.Nst1*, like *Bt1*, directly encodes an ADP-Glc transporter. To investigate this further, we compared the sequence of the *Hv.NST1* and *BT1* proteins with those of other known transporters.

Hv.NST1 Is a Member of the Mitochondrial Carrier Family

Hv.NST1 and *BT1* have no obvious sequence similarities at the amino acid level to NSTs that are found in the Golgi and endoplasmic reticulum of eukaryotes. The latter NSTs belong to the drug/metabolite transporter superfamily of transporters. The proteins most closely related to *Hv.NST1* and *BT1* are members of a family of membrane-located metabolite transporters (Sullivan et al., 1991), most of which are found in the mitochondrial outer envelope. This family is known as the mitochondrial carrier family (MCF), and it contains approximately 2,000 members (58 in the Arabidopsis genome; Picault et al., 2004) and includes carriers for many different sorts of metabolites, including ATP but excluding nucleotide sugars.

The protein sequences of members of the MCF consist of three tandem repeats of approximately 100 residues (Palmieri, 2004; Picault et al., 2004). The

Figure 5. The primary and secondary structures of the ADP-Glc transporter. a, The primary structure of the Hv.NST1 protein showing the sizes and positions of the transit peptide (TP) and the three MCF domains (shaded dark gray and numbered). b, Diagram of the arrangement of helices in the plastid envelope. Helices 1, 3, and 5 contain a conserved motif (PX(D/E)XX(K/R)) that is predicted to cause the helix to kink. c, Comparison of the three MCF domains of Hv.NST1 from the wild-type cultivar Bomi and the corresponding sequences from the wild-type Carlsberg II (Carlsberg) and three of the *lys5* mutants, Risø 13, Risø 29, and Risø 86. Amino acids identical to those in the corresponding domains of Bomi are indicated by a dash. The conserved kink motif is indicated.



structure of one member of the MCF, the bovine ATP/ADP translocator, was recently solved at a resolution of 2.2 Å by x-ray crystallography (Pebay-Peyroula et al., 2003). This protein was shown to have six transmembrane helices (two helices in each of the three repeats) with the conserved sequence containing a Pro residue (PX[D/E]XX[K/R]) creating a kink in every other helix (Fig. 5). The six helices enclose a deep cavity open to one side of the membrane. The transporter is thought to act as a dimer, with one monomer binding a substrate at the inside surface of the membrane and the other monomer binding the second substrate at the outside surface. Transport is proposed to involve flexing of the kinked helices, which transiently opens the base of the cavity forming a channel (Pebay-Peyroula et al., 2003).

Examination of the protein sequence of Hv.NST1 (PROSITE; ca.expasy.org) shows that it contains the three repeated domains (PROSITE no. PDOC00189) characteristic of the MCP family (Fig. 5a). An alignment of the three repeats in Bomi Hv.NST1 is shown in Figure 5c. A search for predicted transmembrane helices in Hv.NST1 (TMpred; www.ch.embnet.org) suggests that there are at least four; two in repeat 2 and one each in repeats 1 and 3. The similarity between Hv.NST1 and the ATP/ADP translocator suggests that it is very likely to have a similar fold. It is therefore likely that each of the three repeated domains in Hv.NST1 comprises a pair of helices,

making a total of six in the protein as a whole (Fig. 5b). The conserved motif (kink motif) responsible for creating a kink in every other helix of the ATP/ADP translocator is also conserved in the three repeats of Hv.NST1 (Fig. 5c).

Amino Acid Substitutions in Hv.NST1 Explain Loss of ADP-Glc Transport Capacity

The *lys5* mutants have aberrant Hv.NST1 proteins with single amino acid substitutions compared to the wild-type proteins. It is likely that these mutations completely or almost completely prevent the transport of ADP-Glc by Hv.NST1. Despite evidence for a low level of ADP-Glc transport in plastids isolated from mutant endosperm (Table III), the starch content of Risø 13 is almost as low as that in Risø 16, a null mutant for cytosolic AGPase activity (Johnson et al., 2003), suggesting that ADP-Glc transport into plastids in the mutants is minimal. To understand why these amino acid substitutions might inactivate Hv.NST1, we examined the conservation of these residues within the MCF and their position and role as predicted from the crystal structure of the bovine ATP/ADP translocator.

The Pro residue in Hv.NST1 that is substituted with a Ser residue in mutants Risø 29 and 86 lies within the conserved sequence (PX[D/E]XX[K/R]) that is responsible for the kink in helix 3 (Fig. 5b). It was predicted that the conserved Pro residues in the

three kinked helices were essential for transporter function (Pebay-Peyroula et al., 2003). The mutations in Risø 29 and 86 that lead to the loss of transporter activity, therefore, provide support for this prediction.

Mutant Risø 13 has a point mutation that results in the substitution of Glu for the conserved Val residue at position 273, predicted to be within transmembrane helix 4. The loss of function caused by this substitution may be explained by a change in hydrophobicity. The hydrophilic residue Glu in the mutant is unlikely to replace functionally the hydrophobic residue Val present in the wild type.

A single nucleotide polymorphism was discovered between the wild-type *Hv.Nst1* genes resulting in the amino acid residue at position 184 in helix 2 in Bomi (Ala) being different from that in Carlsberg II (Gly). This conservative substitution presumably has little effect on the activity of Hv.NST1 since these two wild-type cultivars accumulate starch to the same extent (Table I).

CONCLUSION

We have shown that Hv.NST1 is a plastidial ADP-Glc transporter that is necessary for starch synthesis in barley endosperm and that the gene encoding this protein lies at the *Lys5* locus. The *lys5* mutations cause amino acid substitutions in Hv.NST1 that are likely to disrupt transport capacity. Plastids in the mutants have a reduced capacity to import ADP-Glc, and the mutant grains have increased levels of ADP-Glc and decreased starch contents.

This is the first demonstration, to our knowledge, of the way in which ADP-Glc for starch synthesis is supplied in any cereal endosperm other than maize. Prior to this work, doubts were raised about the existence of BT1-like ADP-Glc transporters in cereal endosperm other than maize. We have shown that a BT1-like transporter, Hv.NST1, is present in barley endosperms, and, thus, it is likely that transporters homologous to BT1 and Hv.NST1 are of widespread or perhaps universal occurrence in graminaceous species.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare*) lines were from the John Innes Centre Germplasm Collection (Risø 13, Bomi, Carlsberg II), the Nordic GenBank, Alnarp, Sweden (Risø 29), and the USDA-ARS National Small Grains Collection, Aberdeen, Idaho (Risø 86). Plants were grown in individual pots in a greenhouse at a minimum temperature of 12°C, with supplementary lighting in winter to give 16-h days. Tissues were used immediately or harvested directly into liquid nitrogen and stored at -80°C prior to use.

Extraction and Assay of ADP-Glc and UDP-Glc

Extraction and assay of ADP-Glc and UDP-Glc were performed as described by Johnson et al. (2003).

Extraction and Assay of Enzyme Activities

Extraction and assay of enzyme activities were performed as described by Burton et al. (2002a).

Extraction and Assay of Starch

Extraction and assay of starch were performed as described by Johnson et al. (2003).

Isolation of Plastids from Developing Barley Endosperm

Approximately 2 g of developing barley endosperms weighing 5 to 20 mg each were used. Plastids were isolated as described for wheat in Burton et al. (2002b), except that the homogenate prior to centrifugation at 150g for 10 min was layered onto a cushion of isolation medium containing 2% (w/v) Nycodenz (Sigma, St. Louis). The supernatant was removed from the pellet that consisted of the isolated plastids.

Assay of Starch Synthesis by Isolated Plastids

Isolated plastids were resuspended in 1 to 2 mL of isolation medium (50 mM HEPES, pH 7.5, 0.8 M sorbitol, 2 mM MgCl₂, 1 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% [w/v] bovine serum albumin). An aliquot of plastids was removed and expelled 10 times through a fine bore syringe needle to rupture the plastids. The incorporation of [¹⁴C] from radioactive precursors into starch was measured as follows: 100 μL of plastids (intact or broken) were incubated with 100 μL of isolation medium containing 0.5 mM ATP and either 3 mM ADP-[¹⁴C]Glc (6 GBq mol⁻¹) or 3 mM [¹⁴C]Glc-1-P (6 GBq mol⁻¹) for 30 min at 25°C. The incubations were carried out in 5-mL tubes and were carefully mixed at 10-min intervals. Reactions were terminated by addition of 2 mL of ice-cold 1% (w/v) KCl in 75% (v/v) aqueous methanol. A 100-μL aliquot of amylopectin in solution (10 mg mL⁻¹) was added as a carrier for starch. After 15 min, the methanol-insoluble glucan polymers were collected by centrifugation at 3,300g for 5 min. The supernatant was discarded and the pellet containing glucans was resuspended in 300 μL of water. These precipitation and washing steps were repeated a total of three times. Scintillation fluid (3 mL) was added to the pellet, and radioactivity was determined by liquid scintillation spectroscopy for 5 min. For each plastid preparation, the rates of starch synthesis were measured in triplicate samples containing intact plastids and triplicate samples containing deliberately ruptured plastids. The rates of starch synthesis that were dependent on plastid intactness were calculated.

For each plastid preparation, the yield of plastids and the contamination of these with cytosolic enzymes were determined by measurement of the cytosolic marker enzyme, alcohol dehydrogenase, and the plastid marker enzyme alkaline pyrophosphatase in the supernatant and plastid-containing pellet fractions. Prior to assay, the plastids in the supernatant and plastid fractions were ruptured by expulsion 10 times through a fine bore syringe needle, and then starch was removed by centrifugation at 16,000g for 5 min. Alcohol dehydrogenase was assayed as described by Cossins et al. (1968). Alkaline pyrophosphatase was assayed as described in Gross and ap Rees (1986), except that 50 mM Bicine, pH 8.9, 12.5 mM MgCl₂, 1.25 mM sodium pyrophosphate, and 50 μL of plastid extract were used. The yield of plastids in these experiments was routinely 15% to 25% for Bomi and 20% to 30% for Risø 13. The contamination of the pellet with cytosolic marker enzyme was less than 0.5%.

Isolation of Plastid Envelopes

Isolated plastids were resuspended in 2 mL of lysis medium (100 mM Tricine, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1.5% (v/v) protease inhibitor cocktail [Sigma]) and expelled 10 times through a fine bore syringe needle to rupture the plastids. After centrifugation at 16,000g for 10 min at 4°C, the envelopes were collected from the supernatant by centrifugation at 100,000g for 1 h at 4°C. The pellet was resuspended in 2 mL of lysis buffer containing 1.2 M NaCl, mixed, and centrifuged at 100,000g as before. The pellet containing plastid envelopes was stored at -70°C until use.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed as described by Burton et al. (2002b).

Scanning Electron Microscopy

Scanning electron microscopy was performed as described by Burton et al. (2002a).

Selection of Risø 13/Risø 16 Double Mutants

A cross was made between Risø 13 and Risø 16, and the resulting F1 grains were allowed to grow and self-fertilize to give a F2 population of grains segregating for the two mutations from Risø 13 and Risø 16 and for plump and shriveled grains. Since we expected a double mutant to have grains that were at least as shriveled as those of the two parents, we selected 21 of the most shriveled grains, grew these, and crossed the plants to both parental lines, Risø 13 and Risø 16. The number of plump and shriveled seeds in the resulting progeny was noted. Four of the plants gave 100% shriveled seeds when crossed to both parents. These lines were designated as putative double mutants.

The genotype of the putative double mutant plants was checked further as follows. First, we rechecked for the presence of the *lys5* mutation from Risø 13 by growing five seeds from each putative mutant, crossing to Risø 13, and noting the number of shriveled seeds produced. Second, to check for the Risø 16 mutation in the putative double mutants, grain from the putative mutants was grown and allowed to self-fertilize. Some of the developing grains from each plant were screened for the presence of the cytosolic small subunit of ADP-Glc pyrophosphorylase. This protein is absent in lines that are homozygous for the Risø 16 mutation (Johnson et al., 2003). Extracts of the grains were subjected to SDS-PAGE followed by immunoblotting. The immunoblots were probed with an antibody to the BT2 protein of maize (*Zea mays*; as described in Johnson et al., 2003). Two of the four putative double mutant lines lacked the cytosolic small subunit of ADP-Glc pyrophosphorylase, confirming that they carried the Risø 16 mutation. They also gave 100% shriveled seeds when crossed to Risø 13, confirming the presence of the *lys5* mutation from Risø 13. Grains from these two double mutant lines were used in subsequent experiments.

Identification of Plastid Envelope Proteins

N-terminal sequencing and Q-TOF MS analysis were performed by the John Innes Protein Sequencing Facility as described by Patron et al. (2002) and by Burton et al. (2002b).

Cloning and Sequencing of *Hv.Nst1*

RNA was purified from endosperm tissues of Bomi using Concert Plant RNA reagent (Invitrogen, Paisley, UK) as described by the manufacturer. cDNA was synthesized using the Gene Racer kit for RACE (Invitrogen). Primers for 3' and 5' RACE were designed to expressed sequence tags found in the public database (top hits using tBLASTn with maize *Bt1*; AL507153, AL506887, and AL506597). All RACE products were cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. Primers to amplify the full coding sequence were used to amplify *Hv.Nst1* from all other lines, and all PCR products were cloned and sequenced as above.

Chromosome Location of *Hv.Nst1*

DNA was extracted from young leaves of the barley-wheat addition lines (kindly provided by David Laurie, John Innes Centre) with the DNeasy plant mini kit (Qiagen, Hilden, Germany). Aliquots of DNA (0.5 µg) from each line were digested with *EcoRI* or *XbaI* and separated on a 0.8% agarose gel. DNA was transferred to Duralon nylon membrane (Stratagene, La Jolla, CA) by capillary transfer and cross-linked using UV light. Membranes were hybridized in Perfect-Hyb-Plus (Sigma, Poole, UK) at 65°C for 12 h with *Hv.Nst1* PCR products (complete coding sequence) labeled with [³²P]dCTP using the RediPrime system (Amersham-Pharmacia, Little Chalfont, UK). Autoradiography was performed at -80°C with Kodak BioMax-MS film and a Kodak BioMax intensifying screen (Eastman Kodak, Rochester, NY).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY560327.

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LITERATURE CITED

- Beckles DM, Smith AM, ap Rees T (2001) A cytosolic ADP-Glc pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. *Plant Physiol* **125**: 818–827
- Burton RA, Jenner H, Carrangis L, Fahy B, Fincher GB, Hylton C, Laurie DA, Parker M, Waite D, van Wegen S, et al (2002b) Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. *Plant J* **31**: 97–112
- Burton RA, Johnson P, Beckles DM, Fincher GB, Jenner H, Naldrett M, Denyer K (2002a) Characterization of the genes encoding ADP-Glc pyrophosphorylase in wheat endosperm. *Plant Physiol* **130**: 1464–1475
- Cao H, Shannon JC (1997) BT1, a possible adenylate translocator, is developmentally expressed in maize endosperm but not detected in starchy tissues from several other species. *Physiol Plant* **100**: 400–406
- Cossins EA, Kopala LC, Blawacky B, Spronk AM (1968) Some properties of a higher plant alcohol dehydrogenase. *Phytochemistry* **7**: 1125–1134
- Denyer K, Dunlap E, Thorbjørnsen T, Keeling P, Smith AM (1996) The major form of ADP-Glc pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol* **112**: 779–785
- Doll H (1976) Genetic studies of high lysine barley mutants. In H Gaul, ed, *Barley Genetics III. Proceedings of the Third International Barley Genetics Symposium*. Verlag Karl Thieme, Munich, pp 542–546
- Doll H (1983) Barley seed proteins and possibilities for their improvement. In W Gottschalk and HP Müller, eds, *Seed Proteins: Biochemistry, Genetics, Nutritive Value*. Martinus Nijhoff, The Hague, pp 207–223
- Emes MJ, Tetlow IJ, Bowsher CG (2001) Transport of metabolites into amyloplasts during starch synthesis. In TL Barsby, AM Donald, PJ Frazier, eds, *Special Publication No. 271. The Royal Society of Chemistry*, Cambridge, UK, pp 138–143
- Giroux MJ, Hannah LC (1994) ADP-Glc pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize. *Mol Gen Genet* **243**: 400–408
- Gross P, ap Rees T (1986) Alkaline inorganic pyrophosphatase and starch synthesis in amyloplasts. *Planta* **167**: 140–145
- Islam AKMR (1983) Ditelosomic additions of barley chromosomes to wheat. In S Sakamoto, ed, *Proceedings of the Sixth International Wheat Genetics Symposium*. Maruzen, Kyoto, pp 233–238
- Islam AKMR, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley chromosome addition lines. *Heredity* **46**: 161–174
- Jarvi J, Eslick RF (1975) Shrunken endosperm mutants in barley. *Crop Sci* **15**: 363–366
- Jensen J (1979) Chromosomal location of one dominant and four recessive high-lysine genes in barley mutants. In *Seed Protein Improvement in Cereals and Grain Legumes*, Vol I. IAEA, Vienna, pp 89–96
- Jensen J, Doll H (1979) Gene symbols for barley high-lysine mutants. *Barley Genet Newsl* **9**: 33–36
- Johnson PE, Patron NJ, Bottrill AR, Dinges JR, Fahy BF, Parker ML, Waite DN, Denyer K (2003) A low-starch barley mutant, Risø 16, lacking the cytosolic small subunit of ADP-glucose pyrophosphorylase, reveals the importance of the cytosolic isoforms and the identity of the plastidial small subunit. *Plant Physiol* **131**: 684–696
- Li H, Sullivan TD, Keegstra K (1992) Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize Bt1-encoded protein. *J Biol Chem* **267**: 18999–19004
- Möhlmann T, Tjaden J, Henrichs G, Quick WP, Häusler R, Neuhaus HE (1997) ADP-Glc drives starch synthesis in isolated maize endosperm

- amyloplasts: characterization of starch synthesis and transport properties across the amyloplast envelope. *Biochem J* **324**: 503–509
- Palmieri F** (2004) The mitochondrial transporter family (SLC25): physiological and pathological implications. *Eur J Physiol* **447**: 689–709
- Patron N, Smith AM, Fahy B, Hylton CM, Naldrett M, Rossnagel BG, Denyer K** (2002) The altered pattern of amylose accumulation in the endosperm of low-amylose barley cultivars is attributable to a single mutant allele of granule-bound starch synthase I with a deletion in the 5' non-coding region. *Plant Physiol* **130**: 190–198
- Pebay-Peyroula E, Dahout-Gonzalez C, Kahn R, Trézéguet V, Lauquin GJ-M, Brandolin G** (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* **426**: 39–44
- Picault N, Hodges M, Palmieri L, Palmieri F** (2004) The growing family of mitochondrial carriers in *Arabidopsis*. *Trends Plant Sci* **9**: 138–146
- Shannon JC, Pien F-M, Cao H, Liu K-C** (1998) Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. *Plant Physiol* **117**: 1235–1252
- Shannon JC, Pien F-M, Liu K-C** (1996) Nucleotides and nucleotide sugars in developing maize endosperms. Synthesis of ADP-glucose in *brittle-1*. *Plant Physiol* **110**: 835–843
- Sikka VK, Choi S-B, Kavakli H, Sakulsingharoj C, Gupta S, Ito H, Okita TW** (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADP-Glc pyrophosphorylase. *Plant Sci* **161**: 461–468
- Sullivan TD, Strelow LI, Illingworth CA, Phillips RL, Nelson OE** (1991) Analysis of maize *Brittle-1* alleles and a defective suppressor-mutator-induced mutable allele. *Plant Cell* **3**: 1337–1348
- Tetlow IJ, Bowsher CG, Emes MJ** (1999) ADP-Glc transporter of the amyloplast. European Patent Office Patent No. WO9947681
- Tetlow IJ, Davies EJ, Vardy KA, Bowsher CG, Burrell MM, Emes MJ** (2003) Subcellular localisation of ADPglucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. *J Exp Bot* **54**: 1–11
- Thorbjørnsen T, Villand P, Denyer K, Olsen O-A, Smith AM** (1996) Distinct isoforms of ADP-Glc pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J* **10**: 243–250
- Tobias RB, Boyer CD, Shannon JC** (1992) Alterations in carbohydrate intermediates in the endosperm of starch-deficient maize (*Zea mays* L.) genotypes. *Plant Physiol* **99**: 146–152
- Ullrich SE, Eslick RF** (1978) Allelism evidence for barley high-lysine, shrunken endosperm xenia (sex) mutants. *Barley Genet Newsl* **8**: 109–113