Theor Appl Genet (1999) 99: 27–36

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Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: correspondence with a *low phytic acid* mutation

Received: 24 August 1998 / Accepted: 19 December 1998

Abstract We sequenced and genetically mapped the *myo*-inositol 1-phosphate synthase (MIPS) genes of maize (*Zea mays* L.) and barley (*Hordeum vulgare* L). Our objective was to determine whether the genetic map positions of these MIPS loci correspond with the location of the *low phtyic acid 1* (*lpa1*) mutations that were previously identified in maize and barley. Seven MIPS-homologous sequences were mapped to positions on maize chromosomes 1S, 4L, 5S, 6S, 8L, 9S and 9L, and a similar number of divergent MIPS sequences were amplified from maize. To the extent that we can compare across different genetic mapping populations, the position of the MIPS gene on maize chromosome 1S is identical to the location of the maize *lpa1* mutation. However, only one MIPS sequence was identified in barley and this gene was mapped to a locus on chromosome 4H that is separate from the barley *lpa1* mutation on chromosome 2H. Although several RFLP markers linked to the barley MIPS gene on chromosome 4H also detect loci near barley *lpa1* on chromosome 2H, our experiments failed to reveal a second MIPS gene that could be associated with the barley *lpa1* mutation. Therefore, genetic mapping results from this study support the MIPS candidate-gene hypothesis for maize *lpa1*, but do not support the MIPS candidate-gene-hypothesis for barley *lpa1*. These opposing results contradict the hypothesis that maize *lpa1* and barley *lpa1* are mutations of orthologous genes, which is suggested by the similar biochemical phenotypes of these mutants. Yet, comparisons of RFLP mapping studies show loci that are homologous between maize chromosome 1S, barley chromosome 4H and barley chromosome 2H, including regions flanking the respective MIPS and/or *lpa1* loci. This putative relationship, between the regions flanking the *lpa1* mutations on maize 1S and barley 2H, also supports the assertion that these mutations are orthologous despite contradictory results between our maize and barley candidate-gene experiments.

Key words Phytic acid · *Myo*-inositol 1-phosphate synthase · Genetic mapping · Maize · Barley

Communicated by G. E. Hart

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Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6 *hexakis*phosphate) is the most abundant storage form of cereal grain phosphorus (P), typically representing 65–85% of total seed P and greater than 95% of the total acid- extractable forms of inositol polyphosphate (Raboy 1997). Trace amounts of "lower" inositol polyphosphates (primarily inositol penta*kis*phosphate) are also found in chromatographic analyses of acid kernel extracts. Compared to the large amount of seed phytic acid phosphorus (PAP), a relatively small portion of seed P is bound to various other organic molecules, such as DNA, free nucleotides, protein, lower inositol polyphosphates, etc. Five percent or less of seed P is found as inorganic phosphorus (Pi).

Chemically induced, recessive mutations that decrease grain phytic acid content have been isolated and genetically mapped in maize (*Zea mays* L.) (Raboy and Gerbasi 1996; Raboy and Ertl, personal communication) and barley (*Hordeum vulgare* L.) (Larson et al., 1998). These *low phytic acid* (*lpa*) mutations have the

potential to alleviate the environmental and nutritional problems associated with grain phytic acid (Ertl et al. 1998). Two types of biochemical phenotypes have been detected when screening for *low phytic acid* mutations in barley and maize. Mutations producing these two biochemical phenotypes are circumscribed by two complementation groups, designated *lpa1* and *lpa2*, in both species.

The most common type of maize or barley *lpa* mutation, designated *lpa1*, exhibits large reductions of PAP and corresponding increases of Pi. Although different *lpa1* mutations show quantitative variations in PAP and Pi, all maize and barley *lpa1* mutants show qualitatively identical chromatographic phenotypes (e.g., HPLC or high-voltage paper electrophoresis). No complementation has been observed in allelism tests between the first maize *lpa1* mutation (*Zm lpa1-1*) and 15 other maize *lpa1* mutations (Raboy, Young, and Cook, unpublished data). Likewise no complementation has been observed between the first barley *lpa1* mutation (*Hv lpa1-1*) and ten other barley *lpa1* mutations. These observations indicate that all the barley and maize *lpa1* mutations, isolated and characterized so far, are allelic to one gene in each species. The genetic and biochemical similarities between maize *lpa1* and barley *lpa1* suggest that these are mutations of orthologous genes.

A second type of mutation (designated *lpa2*) was also identified in both maize and barley. The *lpa2* mutations also show large reductions of PAP and increased Pi. However, *lpa2* mutations differ from *lpa1* mutations in that grains from *lpa2* mutants exhibit highly elevated levels of "lower" inositol polyphosphates (primarily inositol penta*kis*phosphate) compared to the wild-types.

Based on the recessive biochemical phenotypes of the *lpa1* and *lpa2* mutations, we hypothesized that *lpa1* mutations act to interrupt the synthesis of inositol (or some other early step of the phytic acid pathway) and that *lpa2* mutations interrupt later steps of inositol polyphosphate synthesis. The first committed step of *myo*-inositol biosynthesis involves *myo*-inositol 1-phosphate synthase (Loewus 1990). Based on studies reported to-date, this conversion provides the only de novo source of *myo*-inositol in all organisms (Loewus 1990). The gene for *myo*-inositol 1-phosphate synthase (MIPS) has been identified and cloned from yeast (Donahue and Henry 1981; Johnson and Henry 1989) and various higher-plant species (e.g.) Johnson and Burk, 1995; Wang and Johnson, 1995; Ishitani et al., 1996; Smart and Fleming 1993. We speculated that *Zm lpa1* and *Hv lpa1* are mutations of the MIPS gene or perhaps some other gene affecting MIPS expression or activity.

The objective of the present study was to isolate and map the barley and maize MIPS gene sequences and to compare the linkage map positions to those reported for the *lpa1* mutations of maize and barley. These studies provide an important test of our candidate-gene hypothesis that *Zm lpa1* and *Hv lpa1* are mutations of the maize and barley MIPS genes.

Materials and methods

Mapping populations and linkage analysis

The barley mapping populations employed for this study include 140 doubled-haploids derived from the cross of Harrington × Morex (Hayes et al. 1997), and 150 doubled-haploids derived from the cross of Steptoe × Morex (Kleinhofs et al. 1993). Seeds for these populations were made available by the North American Barley Genome Mapping Project, and were kindly provided to us by Dr. Dave Hoffman. The data sets used to map the barley MIPS gene in the Steptoe × Morex populations (results below) were obtained from Map_Data in GrainGenes (http://probe.nalusda.gov:8300/cgi-bin/browse/graingenes). Seeds for the wheat-barley chromosome addition lines (Islam et al. 1981) were kindly provided by Dr. Tom Blake and Dr. An Hang. The sodium azide-induced barley *lpa* mutants were isolated from the Harrington genotype.

This study also employed two sets of 40 maize recombinant inbreds (RIs) derived from crosses of CM37 × T232 and CO159 × TX303 (Burr et al. 1988). Seeds for these maize mapping populations were kindly provided by Dr. Ben Burr. The data sets used to map the MIPS genes in these CM37 × T232 and CO159 × TX303 populations (see Results below) were obtained from Map_Scores in MaizeDB (http://probe.nalusda.gov:8300/cgi-bin/browse/maizedb). The EMS-induced maize *lpa* mutants were isolated from the Early-ACR synthetic population. The Early-ACR genotypes used in this study had been self-pollinated through six generations. The maize *lpa1* mutation was mapped, using F_2 populations derived from crosses of the Early-ACR *lpa1* mutant with proprietary inbred lines, during cooperative research performed with Pioneer Hi-Bred Int. (Raboy and Ertl, personal communication).

Linkage and map analysis of MIPS genes in the barley doubled-haploid populations was performed using the F_2 Backcross model of MAPMAKER version 3.0b (Lander et al. 1987). The Recombinant Inbred Selfing (RI Self) model of MAPMAKER 3.0 was used for linkage and map analysis of MIPS genes in the maize recombinant inbred mapping populations. Residual heterozygosity in the recombinant inbred populations was re-coded with missing data values.

DNA and RNA isolation

Leaf and seed tissues were harvested and immediately frozen in liquid nitrogen, and then stored at −70°C until use. Genomic DNA extractions were essentially as described by Murray and Thompson (1980), except that DNA was precipitated from the aqueous supernatant (using a 0.6 vol of isopropanol) immediately following the first chloroform extraction. Total RNA was isolated using the TRIzol Reagent according to the manufacturer's recommendations (Gibco BRL Life Technologies, Gaithersburg, Md.). All mRNA samples were isolated with a single biotinylated oligo(dT) cellulose selection via PolyAT-tract paramagnetic streptavidin particles (Promega, Madison, Wis.).

PCR amplification and analysis

All PCR amplifications (Saiki et al. 1985) were performed using the GeneAmp PCR System 9600 thermocycler (Perkin-Elmer Cetus, Norwalk Conn.) and *Taq* DNA polymerase (licensed for PCR and supplied by Gibco BRL Life Technologies). PCR reactions were conducted in 50 µl with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1–3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM primer, and 1.5 Units of *Taq*

DNA polymerase. A profile of 33–40 thermocycles (30 s @ 94°, 1 min @ 50–55°, 3 min @ 94°) was used for amplifications. Restriction-site polymorphisms were analyzed by incubating 10-µl aliquots of amplified PCR products with three units of the selected endonuclease and the addition of appropriate reaction buffers provided by the supplier (Promega[1]). The PCR amplification products and restriction fragments were fractionated by 6% polyacrylamide-gel electrophoresis (PAGE) and stained with ethidium bromide.

PCR primers

Degenerate primers (F1051 and R1520, Table 1), designed from previously cloned plant and fungal MIPS sequences, were initially used to amplify related sequences of barley. These primers were selected from conserved MIPS sequence regions of *Arabidopsis thaliana* (Johnson and Burk 1995; GenBank accession U30250), *Brassica napus* (GenBank accession U66307), *Citrus paradisi* (GenBank accession Z32632), *Phaseolus vulgaris* (Wang and Johnson 1995; GenBank accession U38920), *Spirodela polyrrhiza* (Smart and Fleming 1993; GenBank accession Z11693), and *Saccharomyces cerevisiae* (Johnson and Henry 1989; GenBank accession L23520). Primers used for initial cloning of cereal MIPS sequences, MIPS RT-PCR amplification of GenBank sequences, and for the mapping experiments described below, are described in Table 1. These PCR primers were selected using Primer3 (Rozen and Skaletsky 1996, 1997) except for the ZmGSPR1580 gene-specific primer of maize. All other primers used in this study (see rapid amplification of cDNA ends in the following section) are referenced using numbers relative to the translation initiation codon of the *S. polyrrhiza* MIPS sequence (GenBank accession Z11693), which also corresponds with the barley and maize MIPS sequences (GenBank accessions AF056325 and AF056326, respectively) that were determined from experiments described in this study.

RT-PCR and rapid amplification of cDNA ends (RACE)

The 5′ and 3′ RACE PCR methods (Frohman et al. 1988; Frohman 1993) were adopted to amplify overlapping and essentially complete MIPS cDNA sequences, using reagent systems provided by Gibco BRL Life Technologies. The 20-mer RACE primers were designed from maize and barley MIPS genomic DNA sequences (see Results). Negative controls, that omit the reverse transcriptase (for 3′ RACE experiments) and terminal deoxynucleotidyl transferase (for 5′ RACE experiments), were performed in order to assay for genomic DNA contaminations.

The barley MIPS 3′ RACE reaction was performed using a set of nested plus-strand primers positioned at nucleotides 808 and 829. The barley 5′ RACE reverse transcription was performed using a minus-strand primer at position 1080. Nested 5′ RACE minus-strand primers, positioned at nucleotides 1049 and 1019 of the barley MIPS sequence, were used for PCR amplification and re-amplification, respectively. Two sets of maize 3′ and 5′ RACE primers were designed from the two different maize genomic DNAs in order to amplify cDNA corresponding to these two MIPS genomic DNA clones. Two sets of nested plus-strand primers selected at positions 777 and 808 (of each maize genomic DNA clone) were used to amplify and re-amplify 3′ RACE products. Two minus-strand primers were selected at position 1052 (of each maize genomic DNA clone) and used for reverse transcriptions in the 5′ RACE experiments. Two more sets of minus-strand primers positioned at nucleotides 1029 and 1009 (of each maize genomic DNA clone) were subsequently used to amplify and re-amplify the 5′ RACE products, respectively. The Abridged Universal Amplification Primer (AUAP) sequence, GGCACGCGTCGACTAGTAC (Gibco Life Technologies), was employed to amplify unknown sequence ends (appropriately modified) in all RACE experiments.

RT-PCR amplifications were performed using reagents in the Superscript One-Step RT-PCR System (Gibco Life Technologies) and methods recommended by the supplier.

Cloning and sequence analyses

Genomic and cDNA PCR amplification products were cloned using the TA method of the Original TA Cloning Kit (Invitrogen, San Diego, Calif.). Inserts were amplified by PCR using M13R + T7 oligonucleotide primers and size-fractionated by PAGE or agarose electrophoresis. Inserts of predicted size, or larger, were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase PCR Product Sequencing Kit (USB, Cleveland, Ohio) with [α-^{35} S] dATP. Restriction site analyses were performed using Genepro software (Riverside Scientific, Bainbridge, Wash.). Sequence homology searches of GenBank were performed using BLAST (Altschul et al. 1990) provided at the National Center for Biotechnology Information website.

DNA and RNA blot-hybridization analyses

The RNA and DNA gel blots (Southern 1975; Thomas 1980) were prepared using Nytran Plus (Schleicher and Schuell, Keene, N.H.) by methods recommended by the manufacturer. The RNA blots

Table 1 *Myo*-inositol 1-phosphate synthase PCR primer sequences

Name	5′ > 3′ Sequence	Strand	Oligo position[a]
F1051	acxtt(c/t)(a/c)gxtc(c/t)aaggagat	Plus	1051
R1520	tcca(a/t)(a/g)atcat(a/g)ttgttctc	Minus	1520
*Zm*F-85	agcctccttcctcctctcac	Plus	− 85
*Zm*F-F808	gcctgtgtcatggagggtgt	Plus	808
*Zm*F1302	gctcttggctgagctcagca	Plus	1032
*Zm*GSPR1580	gttcccttccagcagctaac	Minus	1580
*Hv*F-73	agagatcgatcgagaggcaa	Plus	− 73
*Hv*R1660	aaagacaaaaccgccaaatg	Minus	1660
*Hv*R1089	caccacgttgctctttgaga	MInus	1089
*Hv*INTF1162[b]	cccaccttctacggacat	Plus	1162–77[b]
*Hv*R1479	catgatgttctccagcatc	Minus	1479

[a] Nucleotides numbered positive or negative from the translation initiation codon (of the monocot MIPS gene)
[b] Position 77 pp upstream of nucleotide 1162, within the 93-bp barley intron

were prepared using 3 μg of mRNA or 15 μg of total RNA. DNA was digested by incubation with endonucleases as recommended by the enzyme supplier. The DNA fragments were fractionated by electrophoresis using 0.7% SeaKem LE (FMC, Rockland, Me.) agarose gels. High-specificity, radioactive DNA probes were prepared using the random priming method of the RTS RadPrime DNA Labeling System (Gibco BRL Life Technologies) and [α-^{32}P]-dCTP. Templates were prepared by PCR amplification of cloned inserts, using the M13R and T7 oligonucleotide primers. Hybridizations were carried out at 42°C with 50% formamide, 5 × SSPE, 0.5% SDS, 200 *u*g/ml of sonicated salmon sperm DNA and 10% dextran sulfate, and up to 2–4 × 10^6 cpm per ml of hybridization solution (as described by the membrane manufacturer). The high-stringency washes included 1 h with 0.1 × SSPE/1% SDS (60°C). BioMax MS films with BioMax intensifying screens were used for ^{32}P autoradiography. The hybridization temperature was decreased to 38°C with 40% formamide for low-stringency DNA-blot analysis and initial exposures were made after washing blots in 2 × SSC/0.1% SDS (50°C).

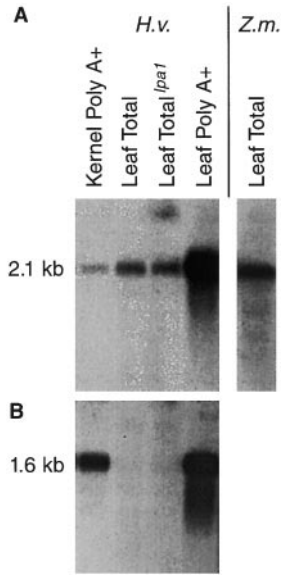

Fig. 1 Northern (RNA)-blot analysis of barley (*H.v.*) and maize (*Z.m.*) *myo*-inositol 1-phosphate synthase (**Panel A**). A control hybridization with a α-tubulin probe is also shown (**Panel B**)

Results

Initial isolation of barley and maize MIPS genomic DNA sequences

The first MIPS genomic PCR product was obtained from barley using a pair of degenerate PCR primers (F1051 + R1520, Table 1) which produced a faint, but discrete, 751-bp amplification product that was larger than the expected size of 470 bp predicted from other plant cDNA sequences. Portions of this 751-bp barley genomic DNA sequence showed a high degree of homology to the other plant and fungal MIPS sequences, particularly that of the aquatic monocot *S. polyrrhiza* (duckweed) which showed up to 27 consecutive nucleotide matches. A corresponding maize genomic DNA clone was obtained using a forward primer designed at position 1089 of the barley MIPS sequence in combination with the *Hv*R1479 primer (Table 1).

The high degree of sequence conservation between the *S. polyrrhiza* and barley MIPS genes suggested that barley and/or maize MIPS sequences might be amplified using non-degenerate oligonucleotides selected directly from available monocot MIPS sequences. The combination of a *S. polyrrhiza* plus-strand primer selected at position 730 and the barley *Hv*R1089 primer yielded two different MIPS sequences from maize; however, no barley MIPS sequences were obtained using these primers. A barley genomic DNA clone, corresponding to these maize sequences, was obtained using the *Zm*F808 primer, designed from these maize genomic DNA sequences, plus the *Hv*R1089 primer.

Amplification of maize and barley MIPS cDNA clones

Northern analysis was performed, using MIPS genomic DNA probes, to determine the feasibility of employing specific RNA preparations for MIPS cDNA amplifications. The barley and maize MIPS genomic DNA clones hybridize to a 2.1-kb transcript (approximate size) detectable on RNA blots prepared from maize and barley leaves harvested at anthesis, and whole barley kernels harvested at 14 days post-anthesis (Fig. 1, panel A). Note that the MIPS hybridization signal was much stronger in the barley leaf poly A + lane compared to the barley kernel poly A + lane (Fig. 1, panel A), whereas the a-tubulin hybridization signals were much closer to equality for these same two lanes (Fig. 1, panel B). These results indicated that the MIPS transcript was present and relatively abundant in our maize and barley leaf RNA preparations. Therefore, complementary DNA was prepared from maize and barley leaf total RNA and used as a template to amplify MIPS cDNAs (see Materials and methods).

The barley 5′ and 3′ RACE experiments yielded partially overlapping cDNAs extending into the 5′ and 3′ untranslated regions (UTRs). The overlapping regions of all barley RACE cDNA clones and genomic DNA clones showed complete sequence identity. The composite sequence of these barley 5′ and 3′ RACE clones is 2152 nucleotides in length (GenBank accession AF056325). Plus- and minus-strand PCR primers (*Hv*F-73 + *Hv*R1660) were designed in the 5′ and 3′ UTRs of this barley MIPS RACE composite and used to amplify a 1733-bp sequence (of predicted size) by RT-PCR. This RT-PCR sequence showed complete sequence identity to the barley MIPS sequence from which the primers were designed. The deduced amino-acid sequence of this barley MIPS cDNA is identical in length (510 amino acids) and 86% homologous by identity (92% homologous by similarity) to the MIPS gene cloned from *S. polyrrhiza* (Smart and Fleming 1993). The predicted molecular weight of this putative barley MIPS enzyme is 56 kDa.

The maize 5′ and 3′ RACE experiments yielded one 5′ RACE clone extending back into the 5′ UTR and three 3′ RACE clones extending into the 3′ UTRs. Sequence divergence among the three different 3′ RACE clones obtained from the same inbred Early ACR plant (data not shown), indicated the likelihood of more than one MIPS locus in the maize genome. One plus-strand primer, designed in the 5′ UTR of the single 5′ RACE clone, was tested in combination with several "sequence specific" minus-strand primers designed from the 3′ UTRs of the three different 3′ RACE clones. The combination of *Zm*F-85 + *Zm*GSPR1580 was used to amplify a 1665-bp maize MIPS sequence, deposited in GenBank (AF056326), using RT-PCR. The deduced amino-acid sequence of this maize MIPS cDNA is identical in length (510 amino-acids) and 86% homologous by identity (92% homologous by similarity) to the MIPS gene cloned from *S. polyrrhiza* (Smart and Fleming 1993). The deduced amino-acid sequence of this maize MIPS cDNA is 94% identical, or 95% similar, to that of the barley MIPS cDNA (GenBank accession AF056325). The predicted molecular weight of this putative maize MIPS enzyme is 56 kDa.

Linkage mapping and genomic DNA analysis of the barley MIPS gene

Two segments of MIPS genomic DNA sequence were amplified from barley. A 452-bp barley MIPS amplification product (Fig. 2, panel A), obtained using the *Zm*F808 + *Hv*R1089 primer pair, showed no detectable restriction-site differences (analyzed by sequence analysis) among several parental genotype mapping populations (e.g., Steptoe, Harrington, and Morex). However, we did localize this barley MIPS genomic DNA amplification product to chromosome 4H (Fig. 2, panel A) by analysis of the wheat-barley chromosome addition lines. A second, 580-bp MIPS genomic DNA amplification product, obtained using the *Hv*F1162 + *Hv*R1479 primer pair, showed a number of restriction-site differences that distinguish Steptoe, Morex, and Harrington, including the *Rsa*I polymorphism shown in Fig. 2, Panel B. The *Rsa*I enzyme cuts the corresponding Steptoe product into 503 + 77-bp fragments, whereas this enzyme produces 262 + 241 + 77-bp fragments from Morex amplification products (Fig. 2, Panel B). This restriction-site polymorphism occurs in an intron immediately following nucleotide position 1350 of the barley MIPS gene. We have determined that this restriction-site polymorphism is 2.1 cM from BCD453b locus (opposite bBE54a, as shown in Fig. 6), on chromosome 4H, in the Steptoe × Morex double-haploid mapping population (Kleinhofs et al. 1993). By our analysis, the three-point map order of MIPS – BCD453b – bBE54a (log-likelihood = 0.00) is only slightly more likely than BCD453b – MIPS – bBE54a (log-likelihood = − 0.10). However, the latter three-point map orders are much more probable than the third possible array (BCD453b – bBE54a – MIPS) which has a log-likelihood of −8.15 in our analysis.

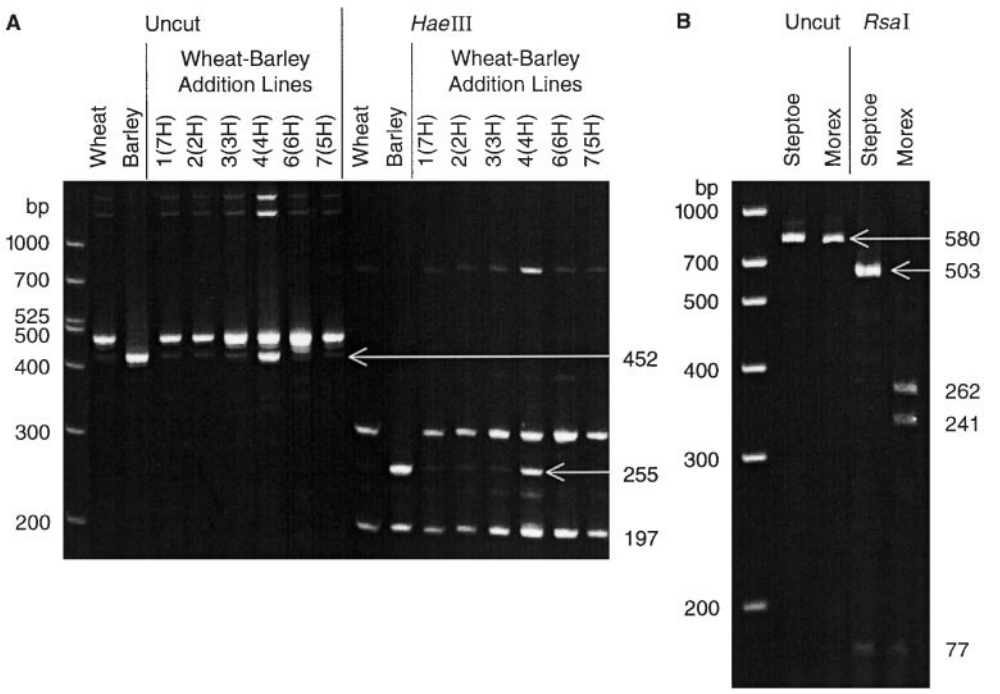

Fig. 2A,B Genomic DNA mapping polymorphisms for the barley *myo*-inositol 1-phosphate synthase gene. **(Panel A)** Wheat-barley chromosome addition lines amplified using the *Zm*F808 + *Hv*R1089 primer pair. **(Panel B)** Barley doubled-haploid mapping population parents amplified using the *Hv*INTF1162 + *Hv*R1479 Primer pair

Genomic DNA-blot analysis suggests that the barley MIPS gene on chromosome 4H is single copy. Barley genomic DNA, digested with six different enzymes, was analyzed under low- and high-stringency hybridization and wash conditions. Both low- and high-stringency analyses show similar signal patterns with a full-length MIPS cDNA probe (Fig. 3, panels A and B), at least for areas not covered by non-specific binding after the low- stringency washes. Non-specific binding was identified as signals spreading across lane spaces where no DNA is present (Fig. 3, panel B). Only one fragment is observed with the *Bam*HI or *Hind*III restriction endonucleases (Fig. 3). An *Eco*RV site in the middle of the barley chromosome-4H MIPS coding sequence produces two fragments (Fig. 3) that have been separately detected using 5′ or 3′ barley MIPS subclones (data not shown). Likewise, an *Eco*RI site in the intron sequence following base 1413 of the barley MIPS coding sequence (data not shown), may account for the two *Eco*RI fragments observed in Fig. 3. Two *Xba*I fragments are observed in the Morex and Harrington genotypes (Fig. 3); however, these segregate as allelic fragments that map to chromosome 4H in the Steptoe × Morex and Morex × Harrington doubled-haploid mapping populations (data not shown). Although three *Bgl*II fragments are detected (Fig. 3), the barley MIPS cDNA sequence includes three *Bgl*II sites. Moreover, two of the *Bgl*II fragments (Fig. 3) are less than 2 kb and the 4-kb *Bgl*II fragment produces a weak signal as though it were partially covered by the MIPS cDNA probe. These results are consistent with our working hypothesis that the barley MIPS gene is a single-copy sequence on chromosome 4H.

Linkage mapping and genomic DNA analysis of the maize MIPS genes

Using our MIPS cDNA clones as probes, we mapped five *Hind*III RFLPs to chromosomes 6S, 9L, 8L, 9S, and 5S (Fig. 4 a and 5 b) and two *Eco*RV RFLPs to chromosomes 1S and 4L (Fig. 4 a and 5 b) in the CM37 × T232 recombinant inbred mapping population. The MIPS RFLP locus on chromosome 1S is 10.8 cM from *umc157* and 9.9 cM from *umc76* with LODs of 4.64 and 5.31, respectively. The log-likelihoods of *MIPS—umc157 − umc76* and *umc157—umc76 − MIPS* are − 2.20 and − 1.93, respectively, relative to the most-likely three-point map order of *umc157—MIPS —umc76* (as shown in Fig. 4 b and Fig. 6). Interestingly, a previous mapping experiment (Raboy and Ertl, personal communication) has shown that that *Zm lpa1* is 10 cM proximal to the *umc157* locus (in a different mapping population), in a region corresponding approximately with the 1S MIPS locus (Fig. 4 b).

The presence of several non-allelic MIPS loci in the maize genome was also evident from the sequence divergence among several genomic and cDNA clones obtained from maize. We identified four different maize MIPS genomic DNA sequences (data not shown), at least two of which were from different loci of the same inbred plant. Substantial DNA sequence and size variation was evident among the corresponding intron sequences of these four MIPS genomic DNA sequences. Likewise, two different 3′ RACE experiments each produced two different cDNA sequences from the same inbred Early-ACR plant (data not shown) using primers designed from the two "non-allelic" genomic DNAs.

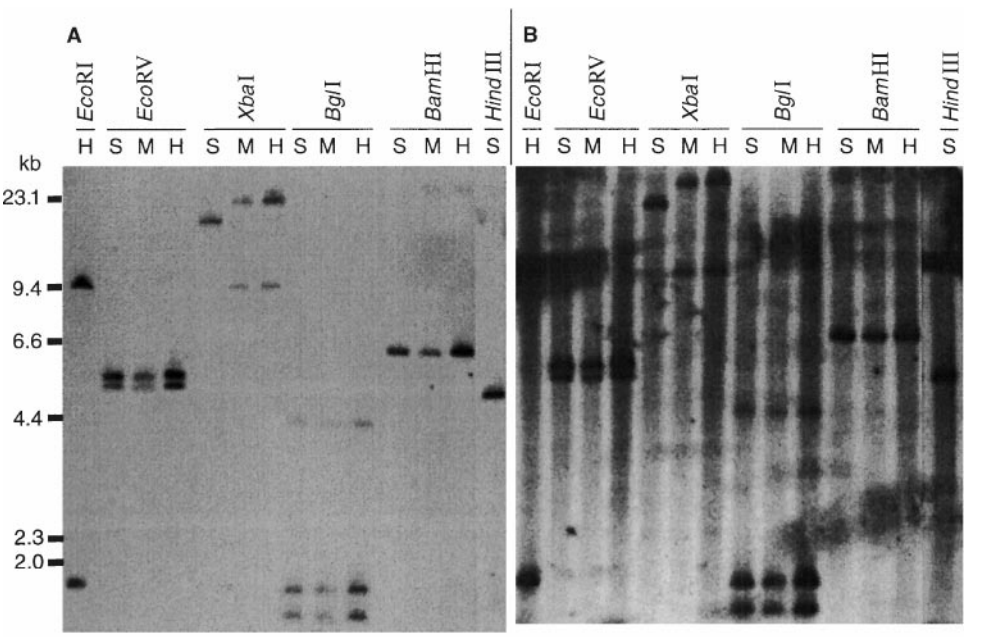

Fig. 3 High-stringency (**Panel A**) and low-stringency (**Panel B**) analysis of a barley genomic DNA blot (Southern) hybridized with a *myo*-inositol 1-phosphate synthase cDNA probe (GenBank accession AF056325, complete CDS). Genotypes analyzed include the barley doubled-haploid mapping population parents; Stepote (*S*), Morex (*M*), and Harrington (*H*). Lambda phage DNA *Hind*III fragments are also shown (left)

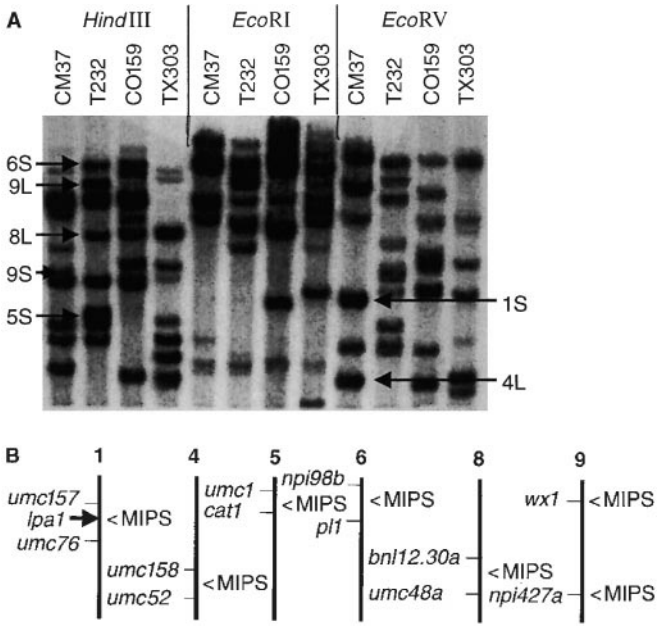

Fig. 4 Panel A Maize genomic-DNA blot (Southern) hybridized with a maize, *myo*-inositol 1-phosphate synthase cDNA probe (GenBank accession AF056326, complete CDS). The two *Eco*RV RFLPs and five *Hind*III RFLPs (indicated by *arrows*) were mapped by genotyping corresponding polymorphisms in the CM37 × T232 recombinant inbred mapping population. Panel B Genetic map of seven maize *myo*-inositol 1-phosphate synthase (MIPS) loci identified by linkage analyses of two *Eco*RV RFLPs (Fig. 5 a) and five *Hind*III RFLPs (Fig. 5 a) in the T232 × CM37 recombinant inbred mapping population

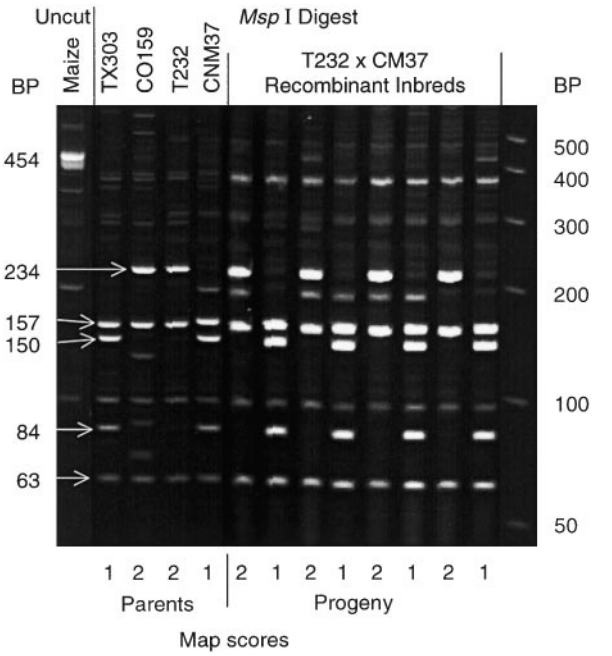

Fig. 5 Genomic DNA-mapping polymorphism and segregation analysis for the maize *myo*-inositol 1-phosphate synthase, gene-specific *Zm*F1302 + *Zm*GSPR1580 amplification product. Sequence lengths of molecular-weight size standards (right) and PCR-amplification/restriction products (left) are indicated. A subset of eight T232 × CM37 recombinant inbred (homozygous) progeny illustrate segregation of the *Msp*1 restriction fragments and the assignment map scores (bottom) used for linkage analysis

PCR primers were designed that amplify specific MIPS sequences mapping to individual loci, including the chromosome-1S locus near *Zm lpa1*. Gene-specific PCR amplification of the chromosome-1S MIPS gene was obtained using the *Zm*F1302 + *Zm*GSPR1580 primer pair (Table 1). The *ZmGSP*R1580 primer was designed in a 3′ UTR sequence that was unique to one of four different 3′ RACE clones obtained from maize leaf tissue. PCR amplification using the *Zm*F1302 + *Zm*GSPR1580 primer pair produced a 454-bp amplification product (Fig. 5) that showed several restriction-site polymorphisms which distinguish CM37 and T232, and/or the TX303 and CO159, recombinant-inbred mapping-population parents. A *Msp*I restriction digest of the 454-bp amplification product yields 234 + 157 + 63-bp fragments from the CO159 and T232 genotypes (Fig. 5). The 234-bp *Msp*I fragment is further digested to 150 + 84-bp fragments from the TX303 and CM37 genotypes (Fig. 5). The latter *Msp*I restriction-site polymorphism is the result of nucleotide variation at position 1431 (a third-nucleotide codon position with no effect on predicted translation) of the last maize MIPS exon. This *Msp*I restriction-site polymorphism cosegregates (Fig. 5) with the MIPS RFLP band (Fig. 4a) mapping to chromosome 1S (at or near the *Zm lpa1* locus; see Fig. 6). This result clearly indicates that the most abundant PCR product obtained using the *Zm*F1302 + *Zm*GSPR1580 primer pair is amplified from the chromosome-1S MIPS locus near *Zm lpa1*.

Comparison of the chromosome-1S MIPS genomic DNA sequences with a complete MIPS coding sequence cDNA

We compared the sequence of the Early ACR MIPS cDNA sequence deposited in GenBank (accession AF056326) to the T232 and CM37 genomic DNA sequences mapped to the chromosome-1S locus near maize *lpa1*. The relationship of this cDNA sequence, amplified using the *Zm*F-85 + *Zm*GSPR1580 primers, to chromosome-1S is suggested by the specificity of *Zm*GSPR1580 to chromosome 1S. Our mapping results (see above) indicated that the *Zm*GSPR1580 primer is specific to the chromosome-1S locus, at least when used in combination with the *Zm*F1302 primer (Table 1). Since the *Zm*F1302 primer was designed from conserved coding sequences, the specificity of *Zm*F1302 + *Zm*GSPR1580 to chromosome 1S probably results from the *Zm*GSPR1580 primer that was designed from a 3′ UTR sequence unique to one of four cDNAs. Although the *Zm*F-85 primer was designed from an "unmapped" 5′ UTR sequence, we surmised

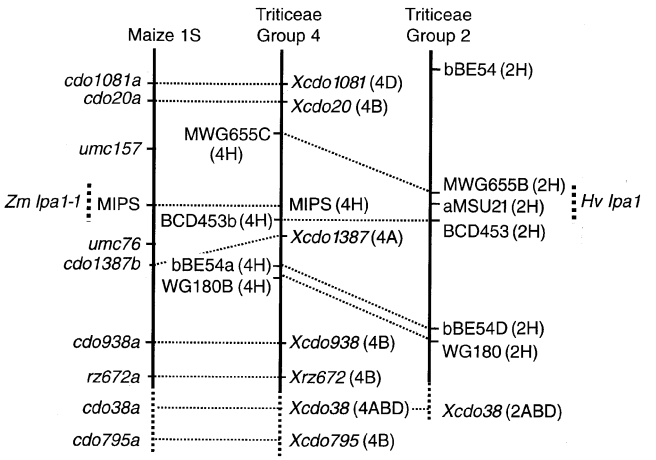

Fig. 6 Comparative genetic map of *low phytic acid 1* mutations (*Zm lap1* and *Hv lap1*) and the *myo*-inositol 1-phosphate synthase (MIPS) loci on maize chromosome 1S and barley chromosomes 4(4H) and 2(2H). Markers that cross hybridize to Triticeae group 2 and 4, or Triticeae group 4 and maize 1S, chromosomes are connected by *dashed lines* (Burr et al. 1988; Ahn and Tanksley 1993; Kleinhofs et al. 1993; Nelson et al. 1995; Van Deynze et al. 1995; Dubcovsky et al. 1996; Langride and Karakousis 1996; Qi et al. 1996). As determined in this study, the most likely three-point map positions are shown for the barley *myo*-inositol 1-phosphate synthase (MIPS) gene, BCD453b, and bBE54a. Likewise, the most probable three-point map positions are shown for the maize MIPS gene, *umc157*, and *umc76*. The *Zm lpa1-1* mutation maps between *umc157* and *umc176* (Raboy and Ertl, personal communication). The barley *lpa1* mutation (*Hv lpa1*) maps within 3 cM of the aMSU21 marker on chromosome 2H (Larson et al. 1998). For each Triticeae marker locus, the chromosome and genome designations (H = *Hordeum*, A = *Triticum monococcum*, B = *Triticum* section Sitopsis, and D = *T. tauschii*) are shown in parentheses

that the *Zm*F-85 + *Zm*GSPR1580 MIPS RT-PCR product may be expressed from the chromosome-1S locus, owing to the specificity of *Zm*GSPR1580. The Early-ACR MIPS cDNA (GenBank accession AF056326) showed only six and four nucleotide differences from the MIPS genomic-DNA amplification sequences that were mapped to chromosome-1S of T232 and CM37, respectively. Two of these nucleotide differences are in the 3′ UTR and the others do not affect predicted translation sequences. These similarities also suggest that the Early-ACR MIPS cDNA, corresponding to GenBank accession AF056326, may be expressed from the chromosome-1S MIPS locus near maize *lpa1*.

Comparative map of maize and barley *lpa1* mutations and MIPS genes

We reviewed other mapping and comparative genome studies to investigate possible evolutionary relationships between chromosomal regions containing the maize MIPS and *lpa1* loci (1S), the barley *lpa1* locus (2H), and the barley MIPS locus (4H). Several mapping projects and comparative studies have documented relationships between maize chromosome 1S and Triticeae chromosome 4 (Ahn and Tanksley 1993; Nelson et al. 1995; Van Deynze et al. 1995; Dubcovsky et al. 1996) as illustrated in Fig. 6. The map positions of the barley MIPS locus (4H) and the maize MIPS locus (1S) are congruent with the linear array of several markers that cross hybridize to genetically linked loci on maize 1S and Triticeae group 4 (Fig. 6). The Triticeae group-4 map is a consensus of several studies, therefore relative genetic distances and perhaps even some gene orders may be slightly distorted, particularly in the dashed map regions near the bottom of this figure. In mapping the barley *lpa1* mutation (Larson et al., 1998) and the barley MIPS gene, we noticed that both loci were closely linked to loci detected by the same BCD453 probe. Further research into this observation revealed several other markers (e.g., MWG655b, bBE54D and WG180) that cross-hybridize to barley chromosome 2(2H) and 4(4H) regions containing the respective *lpa1* and MIPS loci (Kleinhofs et al. 1993; Langridge and Karakousis 1996; Qi et al. 1996). Most notable are the BCD453 and MWG655 loci that are closely linked to the *lpa1* locus on barley chromosome 2H and also linked to the MIPS locus on barley chromosome 4H.

Discussion

The primary objective of this study was to genetically map the maize and barley *myo*-inositol 1-phosphate synthase (MIPS) loci and investigate the candidate-gene hypothesis that these MIPS genes are allelic to the maize and barley *lpa1* mutations. An implicit assumption of this hypothesis was that maize *lpa1* and barley *lpa1* are mutations of orthologous genes.

We mapped seven loci with MIPS-homologous sequences in the maize genome. These loci were mapped to chromosome positions 1S, 4L, 5S, 6S, 8L, 9S and 9L. Our mapping results indicate the maize genome has at least seven MIPS loci. The presence of multiple MIPS loci in the maize genome was also evident by the existence of divergent genomic DNA and cDNA sequences obtained from the same inbred plants. One or more of these different maize MIPS loci are likely to contain a functional MIPS gene. If more than one of these loci contain functional genes, then it is possible that some of these have tissue-specific expression and function. One complete MIPS cDNA coding sequence (GenBank accession AF056326) was obtained from maize leaf tissue. Sequence comparisons suggest, but do not prove, that this expressed MIPS coding sequence may correspond with the 1S MIPS locus. Nevertheless, to the extent that we can compare across different mapping populations, the genetic map position of one MIPS locus, on chromosome 1S, corresponds with the map position of the maize *lpa1*. This observation

supports the candidate-gene hypothesis that *Zm lpa1* is a mutation of a MIPS gene. However, by reasoning of this candidate-gene hypothesis, the six other MIPS loci must either have little or no function in the pathway to kernel phytic acid synthesis since certain single-gene *lpa1* mutations can virtually abolish kernel phytate, without complementation by genes at other loci.

A single-copy barley MIPS gene was mapped to a locus on chromosome 4H. A complete MIPS cDNA coding sequence (GenBank accession AF056325) corresponding to this locus was obtained. Unlike our findings with maize, this barley MIPS gene does not correspond with the barley *lpa1* locus that was mapped to chromosome 2H (Larson et al. 1998). Although several molecular markers closely linked to the barley 4H MIPS gene also detect loci very near the barley 2H *lpa1* mutation, our experiments failed to reveal a second MIPS locus that we anticipated might be near the *lpa1* mutation on barley chromosome 2H. Therefore, our findings do not support the candidate-gene hypothesis that *Hv lpa1* is a cis-acting mutation of a barley MIPS gene. Yet, evidence of evolutionary relationships between maize chromosome 1S and barley chromosomes 4H and 2H provide more support of our assertion that maize *lpa1* and barley *lpa1* are orthologous (Fig. 6). The contradictory results of our MIPS candidate-gene studies, for maize and barley *lpa1* mutations, are not consistent with our hypothesis that maize *lpa1* and barley *lpa1* are orthologous.

The identification of a second barley MIPS gene on chromosome 2H would provide a simple resolution of these contradictory results from our candidate-gene studies of maize and barley. Such a finding would be compatible with our candidate-gene hypotheses and our hypothesis that maize *lpa1* is orthologous to barley *lpa1*. An alternative explanation is that the *Zm lpa1* mutation is allelic to the 1S MIPS gene and *Hv lpa1* is a mutation of some other gene (possibly a "trans"-acting MIPS factor). Another interesting possibility is that *Hv lpa1* and *Zm lpa1* are mutations of orthologous genes not allelic to MIPS. For the latter case, the coincidence of *Zm lpa1* with the MIPS gene on chromosome 1S might be explained by a cluster of genes related to inositol metabolism, or else of course to mere coincidence. Precedence, in grasses, for clusters of genes with related metabolic functions has been established with the cloning of the maize *bx1* gene which is genetically linked to a cluster of four cytochrome P-450-dependent mono-oxygenase genes (Frey et al., 1997) designated *bx2*, *bx3*, *bx4* and *bx5*. More experiments are required to resolve these three possibilities.

Acknowledgements This work was supported, in part, by the U.S. Department of Agriculture, Agriculture Research Service, and in part by the U.S. Department of Agriculture, National Research Initiative Competitive Grants Program Award No. 97-35300-4421. The University of Idaho Agriculture Research and Extension Center (Aberdeen and Parma) also provided support.

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