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Linkage mapping of two mutations that reduce phytic acid content of barley grain

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Abstract This study describes the inheritance and linkage map positions of two *low phytic acid* barley (*Hordeum vulgare*) mutations, *lpa1-1* and *lpa2-1*, that dramatically reduce grain phytic acid content and increase inorganic seed phosphorus (P). Wide-cross, F_2 mapping populations were constructed by mating six-rowed varieties, 'Steptoe' and/or 'Morex', with two-rowed 'Harrington' *lpa* donor lines homozygous for either *lpa1-1* or *lpa2-1*. The barley *lpa1-1* mutation showed normal inheritance patterns, whereas a deficiency of homozygous *lpa2-1*/*lpa2-1* F_2 plants was observed. We identified a codominant, STS-PCR marker (aMSU21) that cosegregated with *lpa1-1* in a population of 41 F_2 plants. The aMSU21 marker was then mapped to a locus on barley chromosome 2H, using a North American Barley Genome Mapping Project (NABGMP) doubled haploid population ('Harrington' × 'Morex'). We determined that *lpa2-1* is located within a recombination interval of approximately 30 cM between two AFLP markers that were subsequently mapped to barley chromosome 7H by integration with the same NABGMP population. Recent comparative mapping studies indicate conserved genetic map orders of several homologous molecular marker loci in maize and the Triticeae species that also show corresponding linkage to the biochemically similar *lpa2* mutations of maize and barley. This observation suggests that barley and maize *lpa2* mutations may affect orthologous genes. No such evidence for correspondence of the phenotypically similar *lpa1* mutations of barley and maize has been revealed.

Key words Barley · Mutations · Phytic acid

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Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate or IP6) is an abundant storage form of phosphorus (P) in seeds and is detectable in most other plant tissues (Cosgrove 1980; Lott 1984; Raboy 1990). Within each species, quantitative analyses of cereal grains reveal relatively constant molar proportions of inorganic P, cellular P, and phytic acid P (Lolas et al. 1976; Batten 1986; Raboy et al. 1991; Feil and Fossati 1997). Genotype and environment (Raboy et al. 1984) significantly affect total P content, and these ratios of various P forms are not the same among all plant species. Barley seeds, for example, store about 60–75% of the total seed P (about 3–5 mg P/g tissue) as phytic acid (V. Raboy, unpublished results),which is deposited in the aleurone and germ tissues (O'Dell et al. 1972). Another 5–10% of the barley seed P is in the free, inorganic form. The remaining portions of seed P are referred to as "cellular P", including forms such as polymerized and free nucleotides, phospholipids, and other phosphorylated compounds such as proteins.

Mutations that reduce phytic acid content of grains, termed "*low phytic acid*" mutations (*lpa*), have been isolated by chemical mutagenesis from maize (Raboy and Gerbasi 1996) and barley. The *lpa* mutations are recessive and translocation of gene products or metabolites does not appear to complement the loss of gene function in homozygous mutant (*lpa*/*lpa*) seeds from heterozygous (*Lpa*/*lpa*) maternal plants. Unlike normal genetic and environmental effects (which predominantly affect total seed P), these *lpa* mutations show

dramatic reductions in the ratio of phytic acid P to inorganic P.

Two types of biochemical phenotypes have been detected when screening for *lpa* mutations in barley and maize. The causal mutations for these two biochemical phenotypes circumscribe two complementation groups that are similar in both species. The first, and most common, type of mutation (designated *lpa1*) shows a decrease in phytic acid P and a molar-equivalent increase in inorganic P. The second type of mutation (designated *lpa2*) also shows a decrease in phytic acid P, but does not show molar-equivalent increases in inorganic P (relative to loss of phytic acid P). Instead, a significant portion of the total seed P remains bound in lower inositol polyphosphates (e.g., *myo*-inositol penta*kis*phosphate or IP5) in the *lpa2* mutants. Nevertheless, all *lpa* phenotypes appear to be the result of single-gene, recessive mutations that result in significant reductions of phytic acid P (i.e., 50% or more) and at least several fold increases in inorganic P (i.e., 5–10 fold for *lpa1* and 3–4 fold for *lpa2*) relative to normal kernels. Therefore, each of these two complementation groups show distinct phenotypes which may be useful separately, or perhaps even combined. These *lpa* mutations have the potential to significantly reduce the environmental and nutritional problems associated with cereal phytic acid (Ertl et al. 1998).

The objective of this study was to examine the inheritance and determine the linkage map positions of barley *lpa1-1* and *lpa2-1*. These are the first barley mutations identified for the phenotypically distinct *lpa1* and *lpa2* complementation groups. The similarity of biochemical phenotypes between the barley and maize *lpa* mutations suggests that the genes containing the barley and maize *lpa1* mutations, and/or genes containing the *lpa2* mutations of barley and maize, may be orthologous. Another important goal of this study was to utilize information from comparative mapping studies between maize and the Triticeae species (Van Deynze et al. 1995) to investigate the hypothesis that genes containing the respective barley and maize *lpa* mutations (e.g., *lpa1* and/or *lpa2*) affect orthologous genes.

Methods

Description of plant genetic materials used to construct *lpa* mapping populations

The barley *lpa1-1* and *lpa2-1* mutations were obtained by sodium azide seed treatment of barley, var 'Harrington' (V. Raboy, unpublished results). The *lpa1-1* allele was the first of ten mutations that showed a decrease (approximately 45% for *lpa1-1*, corrected for total P) in phytic acid P and a near-equivalent increase of inorganic phosphate, with no elevated levels of "lower" inositol polyphosphates. The *lpa2-1* allele was the first of two mutations that showed a decrease (nearly 70% for *lpa2-1*, corrected for total P) in phytic acid P with substantial increases of inorganic phosphate and IP5. The *lpa* donor lines used to develop the mapping populations employed by this study had been self-mated through five generations to produce M5 'Harrington' lines homozygous for *lpa1-1* or *lpa2-1*.

Wide-cross mapping populations were constructed by mating 'Harrington' *lpa1-1* donor lines with both 'Steptoe' and 'Morex' to produce F_2 mapping populations segregating for the *lpa1-1* mutation. A 'Harrington' *lpa2-1* donor line was crossed with 'Morex' only to produce an F_2 mapping population segregating for the *lpa2-1* mutation. 'Harrington' (Harvey and Rossnagel 1984) is the North American malting industry standard of two-rowed barley varieties. Likewise, 'Morex' (Rasmusson and Wilcoxson 1979) is the U.S. malting industry standard of six-rowed barley varieties. 'Steptoe' (Muir and Nilan 1973) is a six-rowed feed barley. These three spring barley varieties represent genetically distinct germplasm groups that show comparatively high levels of molecular genetic polymorphisms with reference to the cultivated barley gene pool (Hayes et al. 1997). These crosses also enabled us to integrate molecular markers linked to *lpa* mutations with the 'Steptoe' × 'Morex' and/or 'Harrington' × 'Morex' maps produced by the North American Barley Genome Mapping Project (NABGMP) as described below.

Genotypic analysis of *lpa* genes

Segregation analysis of the *lpa* mutations (e.g., *lpa1-1* or *lpa2-1*) was conducted by progeny testing each F_2 plant using inorganic P assays (Chen et al. 1956) of ten individual F_3 kernels, as described elsewhere (Raboy et al. 1994). The effects of the *lpa1-1* and *lpa2-1* mutations are large enough that these Mendelian-like seed traits can be scored on an individual kernel basis. Since the effect of *lpa1* and *lpa2* mutations on grain phytic acid are recessive and independent of maternal plant genotype, seeds from heterozygous F_2 plants (*Lpa*/*lpa*) are expected to show a 3:1 ratio of normal (*Lpa*/*Lpa* or *Lpa*/*lpa*) to high inorganic phosphate (*lpa*/*lpa*) content. The F_2 plants were classified as heterozygous (*Lpa*/*lpa*) if at least one F_3 progeny kernel showed high inorganic phosphate (*lpa*/*lpa*).

Molecular marker analyses of *lpa* mapping populations

The molecular markers employed for linkage analysis of the *lpa* mutations included amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) and a sequence-tagged site-polymerase chain reaction (STS-PCR) marker (aMSU21) for the xMSU21 barley genomic DNA clone (Shin et al. 1990). The aMSU21 primers (GGTCTTTCATGTACTACC and CGAGCTCCTGTCGAGG), designed from the xMSU21 barley genomic DNA clone, have been shown previously to amplify an insertion/deletion polymorphism that distinguished the six-rowed varieties 'Robust' and 'Trail' from the two-rowed varieties 'Betzes' and 'Apex' (Shin et al. 1990). The AFLP analyses were performed using a Perkin-Elmer[1] 9600 thermocycler and AFLP reagents (e.g., AFLP Analysis System I, AFLP Starter Primer Kit, and Taq DNA polymerase) purchased from Life Technologies[1] (Gaithersburg, Md.). The AFLP marker loci designations (described in this study) correspond to Keygene (Kg) laboratory codes for combinations of the following *Eco*RI (E) and *Mse*I (M) selective amplification primer sequences:

E37: GACTGCGTACCAATTCACG
M59: GATGAGTCCTGAGTAACTA
E38: GACTGCGTACCAATTCACT

[1] Specific manufacturer or vendor names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others

M47: GATGAGTCCTGAGTAACAA
E40: GACTGCGTACCAATTCAGC
M48: GATGAGTCCTGAGTAACAC

The *Eco*RI and *Mse*I pre-amplification primer pair sequences are AGACTGCGTACCAATTCA and GATGAGTCCTGAGTAAC, respectively. These AFLP marker loci designations are sufficient to distinguish AFLPs described in this study. However, these AFLP primers yield a variety of other amplification products that map to other genomic map positions in barley and other plant species. The corresponding author can provide images or other details regarding the specific AFLP polymorphisms which showed linkage to the barley *lpa* mutations (Results below).

Integration of *lpa* markers into NABGMP maps

Molecular markers showing linkage to the *lpa* mutations were aligned to the NABGMP maps using subsets of two NABGMP doubled haploid mapping populations. The 'Harrington' × 'Morex' (Hayes et al. 1997) data set included 104 markers. The 'Steptoe' × 'Morex' (Kleinhofs et al. 1993) data set included 434 markers, except when we used the subset of 15 doubled haploid lines that included a "skeletal" set of 124 markers described by Mgonga et al. (1994).

Computations for linkage analyses and mapping

Linkage tests between the molecular markers and *lpa* mutations were not necessarily based on random selections of F_2 plants. Homozygous F_2 plants were selected for AFLP analyses (and DNA extractions in general) when genotypic information for the *lpa1-1* or *lpa2-1* mutations were available before the time of analyses. The rationale for this approach was based on the supposition that most AFLP polymorphisms would be scored as dominant markers and that linkage tests would, therefore, be more sensitive when based on homozygous progeny. Calculations of significance for linkage tests and construction of three-point maps between molecular markers and the *lpa* mutations were performed using the F_2 intercross model of MAPMAKER version 3.0b (Lander et al. 1987). Integration of molecular makers linked to *lpa* mutations with the NABGMP doubled haploid mapping populations was performed using the F_2 backcross model of MAPMAKER.

Results

Segregation analysis of the *lpa* mutations

Table 1 shows the segregation ratios for *lpa1-1* and *lpa2-1* in several F_2 mapping populations, as determined by the ten-kernel progeny tests. Tabulations are provided for three- and two-level genotypic classifications (with putative *Lpa*/*lpa* and *Lpa*/*Lpa* plants combined in the latter case), partly because misclassification may have occurred as a result of progeny test sampling. From Table 1, it is apparent that the frequencies of *lpa*/*lpa* F_2 plants were 19.9% and 15.2% in the combined *lpa1-1* populations and the *lpa2-1* population, respectively.

Several other statistics are also relevant to the inheritance of these mutations. The frequencies of homozygous *lpa*/*lpa* F_3 kernels, from heterozygous F_2 plants, were 22.4% and 25.4% in the *lpa1-1* and *lpa2-1* populations, respectively. The overall germination rates were 91.5% and 97.0% for the *lpa1-1* mapping populations derived from crosses of the 'Harrington' *lpa1-1* donor line with 'Steptoe' and 'Morex', respectively. The overall frequency of germination in the 'Harrington' × 'Morex' *lpa1-1* mapping populations was 81.5%.

Mapping *lpa1-1*

The barley *lpa1-1* mutation was initially localized to chromosome 2H by linkage to a dominant KgE38M47 AFLP marker (Fig. 1). At least 20 other polymorphic AFLP markers from the same primer pair of KgE38M47, plus several STS-PCR markers, failed to show linkage with the *lpa1-1* mutation. The KgE38M47 amplification product linked to barley *lpa1-1* was present in the 'Harrington' and 'Morex' genotypes but absent in the 'Steptoe' genotype. A group of 9 *lpa1-1*/*lpa1-1* and 2 *Lpa1*/*lpa1-1* F_2-derived progeny from the 'Steptoe' × 'Harrington' *lpa1-1* mapping population all showed the presence of the dominant 'Harrington' AFLP marker allele. In addition, 3 *Lpa1*/*Lpa1* progeny from the same population lacked the presence of this allele. The linkage of this KgE38M47 marker and the *lpa1-1* mutation, which cosegregated in 14 F_2 lines, is supported by a LOD score of 3.1. This KgE38M47 marker also cosegregated with the ABC252 restriction fragment length polymorphism (RFLP) locus, indicated in Fig. 1, in a subset of 15 doubled haploid lines (Mgonga et al. 1994) from the 'Steptoe' × 'Morex' NABGMP population (Kleinhofs et al. 1993). A LOD score of 4.5 supported this putative linkage between ABC252 and the KgE38M47 marker. The ABC252 RFLP marker is located on barley 2H (Kleinhofs et al. 1993).

The barley *lpa1-1* mutation is also closely linked to an STS-PCR marker, aMSU21, that shows the same 200-bp insertion/deletion distinguishing six-rowed genotypes (e.g., 'Steptoe' and 'Morex') from the two-rowed genotype (e.g., Harrington) as shown by Shin et al. (1990). The codominant, aMSU21 STS-PCR marker cosegregated with 3 *Lpa1*/*Lpa1*, 11 *Lpa1*/*lpa1-1*, and 8 *lpa1-1*/*lpa1-1* F_2 progeny in the 'Steptoe' × 'Harrington' *lpa1-1* mapping population plus 12 *Lpa1*/*Lpa1* and 7 *lpa1-1*/*lpa1-1* F_2 progeny in the 'Morex' × 'Harrington' *lpa1-1* mapping population. The genetic linkage between this STS-PCR marker and the *lpa1-1* mutation (see Fig. 1), which cosegregated over a total of 41 F_2 progeny, is supported by a LOD score of 21. Shin et al. (1990) report that this STS-PCR marker (aMSU21) is on chromosome 2H. We confirmed this by obtaining segregation data from 93 of the 140 'Harrington' × 'Morex' NABGMP doubled haploid lines. The aMSU21 marker cosegregated with the ABC157 marker, which maps to chromosome 2H of the

Table 1 Genotypic frequencies for the barley *low phytic acid* mutations, *lpa1-1* and *lpa2-1*, as determined in several F_2 populations. The expected frequencies, based on the F_2 genotypic ratios of 1 : 2 : 1 or 3 : 1, are shown in parentheses. Chi-square values for differences between observed versus expected genotypic frequencies are also shown

Population	*lpa*/*lpa* progeny	*Lpa*/*lpa*	*Lpa*/*Lpa*	Chi-square (1 : 2 : 1)[a]	*Lpa*/*lpa* or *Lpa*/*Lpa*	Chi-square (3 : 1)[b]
'Morex' × *lpa1-1*[c]	24 (27.25)	51 (54.5)	34 (27.25)	2.28	85 (81.75)	0.52
Steptoe × *lpa1-1*[c]	13 (19.25)	49 (38.5)	15 (19.25)	5.83	64 (57.75)	2.71
Combined *lpa1-1*[d]	37 (46.5)	100 (93)	49 (46.5)	2.60	149 (139.5)	2.59
'Morex' × *lpa2-1*[c]	14 (23)	44 (46)	34 (23)	8.87*	78 (69)	4.70

*Significant deviation from expected genotypic ratio (alpha = 0.05)
[a]Critical value for chi-square distribution with tail area probability of 0.05 and $df = 2$ is 5.99
[b]Critical value for chi-square distribution with tail area probability of 0.05 and $df = 1$ is 3.84
[c]Harrington lines homozygous for *lpa1-1* or *lpa2-1*
[d]Combined data from populations derived from crosses of the Harrington *lpa1-1* donor lines with 'Steptoe' and 'Morex'

'Harrington' × 'Morex' map (Hayes et al. 1997), in these 93 doubled haploid lines.

In summary, no recombination between barley *lpa1-1* and KgE38M47 (near ABC252) or *lpa1-1* and aMSU21 (near ABC157) were detected in our F_2 mapping populations. However, the ABC252 and ABC157 are separated by about 7 cM in the 'Steptoe' × 'Morex' NABGMP population (http://wheat.pw.usda.gov/graingenes.html), and evidence for tight linkage between *lpa1-1* and the aMSU21/ABC157 loci is much stronger than evidence for perfect linkage between *lpa1-1* and KgE38M47/ABC252 loci. Therefore, *lpa1-1* is probably closer to aMSU21 and ABC157 than to KgE38M47 or ABC252 (as suggested by Fig. 1).

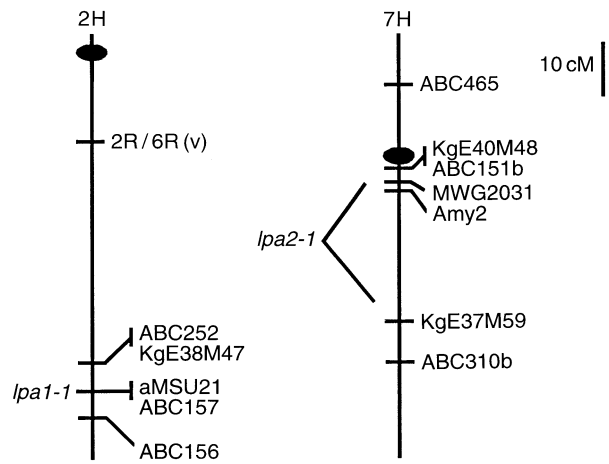

Fig. 1 Linkage map positions for barley *low phytic acid* mutations, *lpa1-1* and *lpa2-1*, which map to chromosomes 2H and 7H, respectively

Mapping *lpa2-1*

Three AFLP primer pairs (KgE37M59, KgE40M48, and KgE40M59) were screened in a population of 27 *Lpa2*/*Lpa2* and 14 *lpa2-1*/*lpa2-1* F_2 lines derived from the cross of 'Morex' × 'Harrington' (*lpa2-1*/*lpa2-1*). Over 30 polymorphic AFLP markers were detected by this survey including 2 that showed linkage with the *lpa2-1* mutation. The KgE37M59 AFLP primer pair revealed a marker that was 20.7 cM (LOD = 4.9) or 10.9 cM (LOD = 13.3) from *lpa2-1* (Fig. 1), depending on whether it was scored as a dominant or codominant marker, respectively. Likewise, the KgE40M48 AFLP primer pair produced a marker that was 20.4 cM (LOD = 3.1) or 25.5 cM (LOD = 4.2) from *lpa2-1* (Fig. 1), depending on whether this band was scored as a dominant or codominant marker, respectively. Differences in AFLP band intensity (i.e., PCR amplification yield), in relation to other monomorphic amplification products visible on the same autoradiograms, were used to deduce the three codominant marker classifications. The most likely three-point map order places *lpa2-1* in between these KgE37M59 and KgE40M48 markers (Fig. 1), which is 2.0 and 2.7 LOD more likely than the other two possible orders.

The genomic map position of the 2 AFLP markers linked to *lpa2-1* was determined by linkage mapping in a subset of 45 doubled haploid progeny from the 'Harrington' × 'Morex' population. The KgE37M59 AFLP was linked to ABC310B (7.9 cM, LOD = 7.7), MWG2031 (23.1 cM, LOD = 2.5), and KgE40M48 (28.0 cM, LOD = 3.2), as shown in Fig. 1. The KgE40M48 AFLP cosegregated with ABC151B (LOD of 13.6 for this linkage) and was linked to MWG2031 (3.7 cM, LOD = 6.6), ABC465 (25.5, LOD = 3.4), and KGE37M59 (28.0 cM, LOD = 3.2), as shown in Fig. 1. The most likely map order of these markers is ABC310B-KgE37M59-MWG2031-KgE40M48-ABC465, which spans 50.2 cM in the 'Harrington' × 'Morex' NABGMP population. Fig. 1 shows the relative positions of these molecular markers, barley

lpa2-1, and the Amy 2 locus (inferred from Kleinhofs et al. 1993 and http://wheat.pw.usda.gov/graingenes.html) on chromosome 7H.

Discussion

Linkage map positions of barley *lpa1-1* and *lpa2-1*

This study provides evidence that at least two loci can be genetically altered to obtain *low* phytic acid phenotypes in barley. A relatively precise genetic map position, on chromosome 2H, was determined for the *lpa1-1* mutation by linkage analysis of the aMSU21 marker (Fig. 1). However, for *lpa2-1*, a number of RFLP probes that have been mapped between the ABC151b and ABC310b loci (http://wheat.pw.usda.gov/graingenes.html) could be employed to refine the linkage map position of this mutation. In any case, the genetic map positions of these mutations may lend clues for the identification of the genes required for grain phytic acid synthesis.

Inheritance of *lpa* mutations

Maize *lpa1-1* male gametes show a 10% transmission deficiency (V. Raboy, unpublished results). However, we observed no significant deficiency of homozygous mutant F_2 plants or F_3 kernels in populations segregating for the barley *lpa1-1* mutation.

The frequency of homozygous *lpa2-1*/*lpa2-1* F_2 barley plants (15.2%) was significantly less than expected based on 1:2:1 or 3:1 segregation ratios (Table 1). However, the observed frequency of *lpa2-1*/*lpa2-1* F_3 progeny kernels (25.4%), from heterozygous F_2 plants, closely approximate the expected value. The overall germination rate for this population (81.5%) was at least 10% lower than would be expected from a cross of normal 'Harrington' and 'Morex' varieties. These data suggest, indirectly at least, that the segregation distortion observed for the barley *lpa2* mutation is caused by a reduction in the germination rate of homozygous *lpa2-1*/*lpa2-1* seeds. However, the genotypes of seeds that failed to germinate were not determined. Observations of maize *lpa2-1* also suggest that this mutation may also show germination problems (results not published), although this has not been rigorously tested.

Comparative mapping studies related to barley and maize *lpa* mutations

One objective of this study was to utilize information from comparative mapping studies between maize and the Triticeae species (Van Deynze et al. 1995) to test the hypotheses that genes containing the respective barley and maize *lpa* mutations (e.g., *lpa1* and/or *lpa2)* are orthologous. The maize *lpa1-1* and *lpa2-1* mutations map to separate loci on chromosome 1S (V. Raboy, unpublished results). Maize *lpa1-1* maps approximately 10 cM from *umc157* (proximal relative to the centromere), whereas maize *lpa2-1* is linked to *umc167* and is distal (relative to the centromere) from the TB1Sb B-A translocation breakpoint.

At least five oat cDNA clones detect loci on maize 1S (e.g., CDO1081, CDO20, CDO1387, CDO38, and CDO938) flanking the approximate linkage map position of maize *lpa1* and detect corresponding loci (i.e., *Xcdo1081*, *Xcdo20*, *Xcdo1387*, *Xcdo38*, and *Xcdo938*) and map order on Triticeae chromosome 4 (Van Deynze et al. 1995). However, we have determined that the barley *lpa1-1* locus maps to a region of chromosome 2H, which shows evidence of correspondence with other sections of the maize genome including homeologous segments of maize chromosomes 2 and 10 (Van Deynze et al. 1995). We find no evidence, based on comparative mapping studies, that supports the hypothesis that barley *lpa1-1* and maize *lpa1-1* are orthologous genes.

The approximate location of maize *lpa2* is between the aforementioned group of cdo loci (near maize *lpa1-1*) and several other loci in maize (e.g., *cdo464 A*, *cdo595*, *bcd98 A*, *bcd98G* and, *bcd98 M*) that also show corresponding locations (e.g., *Xbcd98*, *Xcdo464*, and *Xcdo595*) and map order on Triticeae chromosome 7 (Van Deynze et al. 1995). Based on these comparative mapping studies, our hypothesis was that the barley *lpa2* would map to 7H or 4H. This study shows that barley *lpa2-1* does map to chromosome 7H and is congruent with the linear array of RFLP markers mapped with three clones (namely BCD98, CDO464, and CDO595) that hybridize to homologous loci of maize 1S and barley 7H. This result lends support to the hypothesis that the barley and maize *lpa2* genes are orthologous. However, the map positions for the barley and maize *lpa2* mutations have not been precisely determined, and the correspondence of homologous markers showing synteny with these mutations is not yet very compelling.

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