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Full Length Research Paper

Identification of PCR-based DNA markers flanking three low phytic acid mutant loci in barley

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Phytic acid (PA) is the most abundant form of phosphorus (P) in cereal grains. PA chelates mineral cations to form an indigestible salt and is thus regarded as an antinutritional agent and a contributor to water pollution. Grain with low phytic acid (lpa) genotypes could aid in mitigating this problem. In barley, more than 20 lpa mutant lines have been isolated, representing at least 6 different genetic loci. These mutants have significantly reduced levels of seed PA, which are largely replaced by inorganic P, a form readily digestable by animals. Use of lpa lines in breeding has proved a practical approach for improvement of phosphorus nutrition in barley. Efficient utilization of these loci in marker-assisted selection breeding programs requires identification of closely-linked, high-throughput molecular markers. Here we report development of flanking, PCR-based markers for 3 major lpa loci in barley: lpa1-1 (M422), lpa2-1 linked locus (M640), and a locus linked to the myo-inositol 1-phosphate synthase (MIPS) gene (M678). In addition, marker position accuracy in the MIPs region has been improved by detection and elimination of marker redundancy.

Key words: Barley breeding, SSR marker, low phytic acid (lpa), grain nutrition.

INTRODUCTION

In barley (*Hordeum vulgare* L.) and other small grains, approximately 70-80% of total seed phosphorus (P) is stored as phytic acid (PA; *myo*-inositol-1,2,3,4,5,6-hexakisphosphate) (Ockenden et al., 1997; Raboy, 1997). PA plays critical roles in seed development and germination, DNA repair and mRNA export (Hanakahi et al., 2000; York et al., 1999), cell signaling (Menniti et al., 1993; Sasakawa et al., 1995; Lemtiri-Chlieh et al., 2000) and antioxidation (Graf et al., 1987). PA is not desirable for grain nutrition since it chelates essential cationic minerals, including calcium, iron, and zinc, forming a mixed salt known as phytate and reducing availability of minerals to human and non-ruminant animals. PA is the primary storage form of P in cereal grains; however, phytate is indigestible to non-ruminant animals, resulting in dietary deficiency of P and accelerated eutrophication of waterways (Erdman, 1981; Sharpley et al., 2001). Nutrient impacts of phytate have prompted development of low phytic acid (lpa) mutants in crops such as maize, barley, rice, and soybean (Raboy et al., 2000; Shi et al., 2005; Larson et al., 1998; Larson et al., 2000 and Wilcox et al., 2000). These loss-of-function mutations decrease synthesis of PA from P; consequently, reductions in PA are compensated for by near-equivalent increases in bioavailable forms such as inorganic P (Pi) (Raboy et al., 2000). Thus, detrimental effects of phytate are alleviated without affecting overall seed P levels. Availability of these mutations and the potential benefits of their deployment have made development of *lpa* cultivars a key objective in feed barley breeding programs, providing further impetus to explore the genetics of this trait.

Mutations causing *lpa* can occur at any of the several steps in a complex biosynthetic pathway. Synthesis of PA requires conversion of glucose-6-phosphate to inositol-3-phosphate (Ins (3) P_1), catalyzed by *myo*-inositol 1-phosphate synthase (MIPS). The Ins ring is subsequently phosphorylated to synthesize PA (Loewus, 1990). Since the MIPS reaction is the sole source of Ins, which is necessary for all downstream reactions, mutation and re-

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Abbreviations: cM; Centi-Morgan, **PA;** phytic acid, **MIPs;** *myo*-inositol 1-phosphate synthase, **Pi;** Inorganic phosphate, **STS;** Sequence-tagged-site, **SSR;** Simple sequence repeat, **RFLP;** Restriction Length Polymorphic Fragment, **ISSR;** inter simple sequence repeats and **Ins;** Inositols.

Table 1. Primer pairs for PCR-based markers tested for polymorphism between barley cvs. Harrington, Steptoe and Morex Sequence information was obtained from the GrainGenes website (http://wheat.pw.usda.gov). Italicized marker names are wheat SSRs; all others are barley SSR and STS markers. Marker names in bold exhibited polymorphism.

Locus	Marker	Forward sequence	Reverse sequence
	MSU21	TGGTCTTTCATGTACCTACC	TGTGTCATCAAGCACAACCA
	ABC153	TTCATCATCATCGTCATCGTG	CCTCTGCCGCTGGAACTA
	ABC252	CACAAGGCTCAAAACATAAC	AAGCTCACCAAGTCCCAGTC
	ABC165	CAATGACTTCAAGGGGTCTG	TCCATACCATTCCCATCTAA
	EBmag793	ATATATCAGCTCGGTCTCTCA	AACATAGTAGAGGCGTAGGTG
	EBmac415	GAAACCCATCATAGCAGC	AAACAGCAGCAAGAGGAG
	HVCSG	CACTTGCCTACCTCGATATAGTTTGC	GTGGATTCCATGCATGCAATATGTGG
	Bmac0144b	TACGTGTACATACTCTACGATTTG	ACTTATTCTGCATCCTGGGT
lpa1	EBmatc0039	TAGTCTCTTCATTTATACCATCACC	CATGCTGATCCCCCTTCT
	Bmac0216	GTACTATTCTTTGCTTGGGC	ATACACATGTGCAAAACCATA
	ABG317	CATGATGGGTCAAGCTCTGT	AACTCTGGGTGGTTTGTGAA
	cMWG660	CTGAACCCACAAGAGCAGAA	CCCAGCCAAAGCTGGTTTTT
	WMC175	CTCAGTCAAACCGCTACTTCT	CACTACTCCAATCTATCGCCGT
	MWC243	CGTCATTTCCTCAAACACACCT	CCGGCAGATGTTGACAATAGT
	Barc59	GCGTTGGCTAATCATCGTTCCTTC	AGCACCCTACCCAGCGTCAGTCAAT
	Barc101	GCTCCTCTCACGATCACGCAAAG	GCGAGTCGATCACACTATGAGCCAATG
	Cfd73	GATAGATCAATGTGGGCCGT	AACTGTTCTGCCATCTGAGC
	Bmag0011	ACAAAAACACCGCAAAGAAGA	GCTAGTACCTAGATGACCCCC
	MWG2031.2	TGTGACCTGTCAGACTGTTCAAGTT	AGCCAAGCATATCCTTCACTGACTA
	MWG889	CCCTGAATTCACGCGTTATT	GAATGAGCAACTACCGCATA
	Bmag120	ATTTCATCCCAAAGGAGAC	GTCACATAGACAGTTGTCTTCC
	GBM1126	AGCAGATGATTCCCCAGATG	GCCCACGGTGTAGATGTCTT
	WMG2259	TGATGGGTTCGCAAAGACG	TCCCTCTATGACATGGGCG
	GBM1492	GGAAGGAGACGAACACCAAA	AGGAGATCGAGCACGTAGGA
lpa2	GBM1419	CGTCACGCCACTCACCTC	CTTGAAGTCGGAACCCATGT
	GBM1174	TCTGGAAGAGGAAGGTGAGC	TTCCTCTTACCGTTCTTCGC
	ABG320	GATCCAACAGCAAGGAAAGA	AGACGAGTGGACACATGATG
	Bmag4	GTTTCCCATGCGACGTTC	GATGACGATTGATAGGTGT
	Bmac135	ACGAAAGAGTTACAACGGATA	GTTTACCACAGATCTACAGGTG
	Bmac31	AGAGAAAGAGAAATGTCACCA	ATACATCCATGTGAGGGC
	EBmac565C	ATTTGAATGTCCAACAGAATC	AATTGATAAGTTACTGACACACG
	Bmac0035	TCTCATCATTTTTTTGGGTGG	TGTGACCAAATACAAGAGGCC

duced expression of MIPS causes reduced PA synthesis. The MIPS gene, which exists as a single copy in barley (Larson and Raboy, 1999), has been mapped to chromosome 4H and M678 is a mutant closely linked to this gene.

Besides MIPS, other lpa genotypes result from mutation of kinases in later steps of the PA pathway. More than 20 lpa mutants have been identified in barley, representing at least 6 different loci, more than any other species to date (Hu et al. manuscript submitted). Barley lpa1-1 (M422) and lpa2-1 (M1070) have been mapped to chromosomes 2H and 7H, respectively, and result in 50 and 70% reductions in PA (Larson et al., 1998). In lpa1-1, PA is replaced by a molar equivalent of Pi; in lpa2-1, PA is replaced by both Pi and inositol pentakisphosphate, an intermediate in PA biosynthesis. Barley lpa3-1 (M635) and M955 are non-allelic but linked on chromosome 1H, and exhibit 75 and 95% reductions in PA, both with a corresponding increase in Pi. Other mutants include M640 on chromosome 7H (non-allelic to lpa2-1), and M499 and M2080, both of which are unmapped and could be non-allelic to identified loci (Hu et al. manuscript submitted). Pyramiding of these genes could be useful in generating cultivars with minimal PA.

Since PA is inversely related to Pi, lpa mutants result in a High Inorganic P (HIP) phenotype, which can be identified in a laboratory assay. Since different lpa mutations may have different effects on agronomic traits, combinations of different mutant genes could potentially optimize agronomic benefits in plants. Efficient use of lpa genes in breeding and selection, however, requires a rapid and reliable method for differentiation of individual loci be-

Table 1. Contd.

	Bmag369	CACTAGGCACCAATGACTG	ATCGAAAATCTTAGCTTTGG
	EBmac757	GTGTCTTTTTCACTTCCTTTG	TCTTCACTGTTGAGATGATGA
	EBmac755	AGCCTTGTGTATCAGGACA	CTGCTGGTGTTCTCTAAAAGT
	Bmac0064	CTGCAGGTTTCAGGAAGG	AGATGCCCGCAAAGAGTT
	Bmac582	GCCACATATGCACCCTAGTG	CATGGGGTAGTTTGTGCCTT
	Bmag217	AATGCTCAAATATCTATCATGAA	GGGGCTGTCACAAGTATATAG
	MWG799	TGCAAACTTGATGGCAGGCC	TCGGCGGCCTTGAGGTTGC
	Bmag767	AACTTACCTTCATATGTTGTGG	GAACACTATGATTCCATACGTC
	AWBMS0022	CCACTTCAAAGGCTTCCACA	CCGGAGAGTTGCTAATCT
	Bmac375	CCCTAGCCTTCCTTGAAG	TTACTCAGCAATGGCACTAG
	ABG472	CCGCGTACGCGAATCTGAGT	GCCCAGCTAGGTCGACAATA
	EBmac701	ATGATGAGAACTCTTCACCC	TGGCACTAAAGCAAAAGAC
	EBmac635	TGCTGCGATGATGAGAACT	TAGGGTAGATCCGTCCCTATG
	BCD453b	AGATTTTGTACAACTCAACGGATATCA	AGCTCAAGCCTATTAGGATTCGG
	EBmac679	ATTGGAGCGGATTAGGAT	CCCTATGTCATGTAGGAGATG
	EBMac788	TAACTTACTTTATATCCATGGCA	ATGATGAGAACTCTTCACCC
	Bmag138b	ACCAGGAGGAATGAGAGAG	AATAAACCTTGAGACGATGG
	Bmac577	TCATACAGAAGCCCACACAG	TGCATGTTCATTCTAGACAGG
	EBmag781	CTATTTTCTAATGCTTGGACC	TGTCTAGTTCATCATCATTGC
MIPS	EBmac658	GTATGCAAGTGTAGGTGTGTG	CATGGGTTTACCCACATAC
	GBM1509	CAATCGTTGTCCAGAACCCT	GGCCGACAAATATGCTTCAT
	Bmac84	CTTGTGCCCTTTGATGCAC	CATAACTTGAGGATGTGTGTGACA
	GBM1299	GATCCCCCTAAAAGCAGACC	CTGCCTAGTCCCTGCATCTC
	GBM1220	GCTACCAGAACCCAGGAACA	TGAGCAACCTGAAACTGTCG
	GBM1448	GTATGACACCCGATCCATCC	CAAAATTTGGGACCTGAGGA
	GBM1338	ACGCACAGATACGTACACGC	GCCCCTCCTAGAACACAACA
	Bmag0714B	ATTCCTTATAGAGACACACGC	TTCTCTCCAACAATAAGAAGC
	EBmac691	TTAACAGAGGGCATTGGT	TCCTTTTCTCCATTTGAGTT
	Bmac181	ATAGATCACCAAGTGAACCAC	GGTTATCACTGAGGCAAATAC
	HVM68	AGGACCGGATGTTCATAACG	CAAATCTTCCAGCGAGGCT
	WMS6	CGTATCACCTCCTAGCTAAACTAG	AGCCTTATCATGACCCTACCTT

cause phenotypic assays can not distinguish individual plants for their genotype compositions. Although several lpa-linked RFLP, SCAR and ISSR markers have been identified (Larson et al., 1998; Roslinsky et al., 2007), high-throughput performable and regular PCR-based flanking markers are necessary to reduce undetected recombination between the gene and marker and to facilitate marker-assisted selection. In this investigation, it was reported that identification of flanking, PCR-based markers for 3 major lpa loci: lpa1-1, M640 in the lpa2 region, and the M678 in the MIPs gene region. Those markers are particularly in seedling stage selections in breeding program.

MATERIALS AND METHODS

Plant material

Mutations were induced via sodium azide treatment of barley cv. Harrington using methods described elsewhere (Nilan et al., 1973). Mapping populations were developed by crossing mutant lines M 422 (lpa1-1), M640 (lpa2 region) and M678 (MIPs region) to 'Steptoe.' An additional populations of M640 x 'Morex' and M678 x 'Morex' were also developed and used to detect additional polymerphism at the lpa2-1 locus. Populations were advanced in field plots in Aberdeen, ID and seeds were selected from F3 families for homozygosity testing of phosphorus phenotype in 20 individual seeds of each family. Pooled tissue sample from 20 homozygous seedlings of each family was used to represent the corresponding F2 individuals in DNA extraction and in genetic analysis.

Genotypic analysis

Homozygosity of F3 families at each mutant locus was determined using the single-seed HIP assay, in which Pi is detected colorimetrically (Chen et al., 1956; Raboy et al., 2000). A deep blue color indicated a high concentration of Pi, characteristic of a homozygous mutant genotype. The lpa mutations are recessive (Larson et al., 1998); thus, a 1:2:1 phenotypic ratio is still expected in seed from heterozygous plants. F2 plants were harvested individually and the harvested seeds were F3 families. PA content was measured in 20 seeds per F3 family and families were considered homozygous if assay results were uniform.

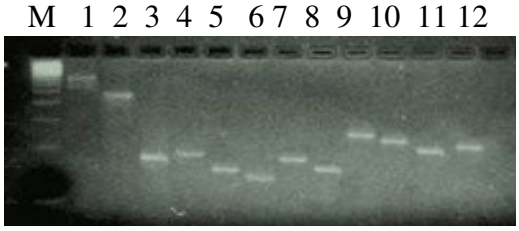

Figure 1. Polymorphisms between DNA samples of barley cvs. Harrington and Steptoe for 6 SSR markers flanking 3 lpa loci. M: 100 bp DNA ladder (Bio-Rad). Odd-numbered lanes were Harrington DNA; even-numbered lanes are Steptoe. PCR products from 2 DNA samples using the same primer pairs were loaded side by side. The order of DNA markers from left to right is MSU21, Bmag415, EBmac701, Bmag714B, Bmag120, and AWBMS0022.

Table 2. Mapping results of flanking DNA markers at 3 *lpa* mutant loci. In each locus, the mapping populations and number of homozygous families were specified.
Proximal and distal markers and their genetic distances from each locus were reported.

Locus area	lpa1 (2H)	M640 (7H)	M678 (4H)
Mutant allele and mapping population used	M422 x Steptoe*	M640 x Steptoe M640 x Morex*	M678 x Steptoe
No. of Homozygous family used	188	51	86 for EBmac701 41 for Bmag714B
Proximal marker	EBmac415	Bmag120	EBmac701
Recombinant	28	7	16
Genetic distance	28/188 = 14.9 cM	7/51 = 13.7 cM	16/86 = 18.6 cM
Distal marker	Msu21	AWBMS0022	Bmag714B
Recombinant	18	6	8
Genetic distance	18/188 = 9.69 cM	6/51 =11.8 cM	7/41 = 17.0 cM

SSR and STS analysis

DNA was extracted using a cetyl trimethylammonium bromide (CTAB) protocol essentially as described elsewhere (Jackson et al., 2006). Molecular markers known to be near *lpa* loci were selected based on previous mapping information and the 2005 bar-ley consensus map (GrainGenes, http://wheat.Pw.usda.gov; Larson et al., 1998; Ramsay et al., 2000; Roslinsky et al., 2007; Varshney et al., 2007). Marker names and sequences screened in this study are summarized in Table 1. Primers of MSU21 amplifying non-specific bands were redesigned to generate simpler banding patterns using Primer3 program (http://frodo.wi.mit.edu) in these cases, the new primer sequences are shown under the same name in Table 1. All markers are either STS or SSR; several are homologous to RFLP or ISSR markers of the same name.

PCR reaction conditions were uniform for all markers, with each 25 µl reaction volume containing 50 ng template DNA, 1 µl each primer (10 µM), 2.5 µl 10X buffer containing the manufacture recommended Mg concentration, 1 µl dNTPs (2.5 mM for each nucleotide), and 1 U Taq polymerase (RedTaq, Sigma, St. Louis, MO). The thermal profile was 94°C for 3 min; 39 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a 4°C hold. Reaction products were analyzed on 3% SFR high resolution agarose gels (Amresco, Solon, Ohio) stained with ethidium bromide and run in1X TAE buffer. A 100 bp DNA ladder (Bio-Rad, Hercules, CA) was used as size marker in each gel. Polyacrylmide gel electrophoresis (PAGE) was conducted according to the protocol published previously (See et al., 2002).

Linkage analysis

Banding patterns were scored on each homozygous family and compared to the genotypes of both parents. Mutant or wild-type scores were also compared to phenotypic results from the HIP colorimetric assay for each individual family. Genetic distances between molecular markers and lpa loci were based on the percentage of recombinant phenotypes within each mapping population. Positions of flanking markers were determined based on the recombinant patterns detected in the individuals of mapping populations and compared to the published barley genetic maps.

RESULTS

Marker polymorphism

Although markers were selected based on known proximity to mutant loci, only a fraction of the primers evaluated were polymorphic between Harrington and Steptoe. For lpa1, 17 primer pairs were screened, including 12 barely markers and 5 wheat SSRs located in the same

Table 3. Comparison of primer sequences of EBmac701 and EBmac788. Bold letters indicate the same primer sequence in 2 markers. Italicized letters indicate the overlapping sequence in primers of the 2 markers. Information in this table was obtained from the GrainGenes database.

Primer name	Forward sequence	Reverse sequence	Product size	Repeat domain
EBmac701	ATGATGAGAACTCTTCACCC	*TGGCA*CTAAAGCAAAAGAC	149	$(AC)_{23}$
EBmac788	TAACTTACTTTATATCCA*TGGCA*	ATGATGAGAACTCTTCACCC	168	$(TG)_{23}$

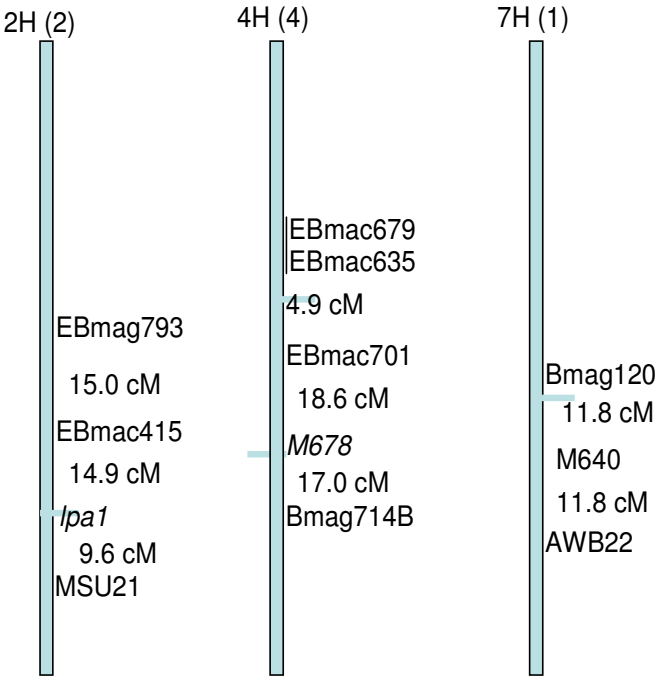

Figure 2. Genetic linkage maps of 3 barley lpa loci, showing flanking marker loci and genetic distance (cM) distances. Names of lpa loci are in bold. Markers and genes in the maps only illustrate the relative positions on chromosomes but not the actual locations.

in the same region of the corresponding wheat chromosome (Somers et al., 2004) (Table 1).

Three markers (MSU21, EBmag793 and EBmac415) were polymorphic (Figure 1). Preliminary test results using 44 individual DNA samples indicated that EBmag793 and EBmac415 are on the same side of the mutant locus but the former was further away (data not shown). MSU21 detected totally different recombinants compared to EBmac415, indicating that it was on another side of the gene. Therefore, only EBmac415 and MSU21 were used as flanking markers for genetic mapping in the selected populations.

Twenty-four primer pairs were evaluated for M640; four were polymorphic (Bmag0011, Bmag120, GBM1419, AWBMS0022). Mapping data indicated that Bmac0011 and Bmag120 were on the same side of the M640 gene but Bmac0011 was farther away. GBM1419 was mapped on the same location as Bmag120. Therefore, Bmag120 was selected as a flanking marker on one side of the gene. AWBMS0022 was the only marker that detected a different recombination pattern from the other 3 markers. Bmag120 and AWBMS0022 were selected as flanking markers for the M640 gene. Polymorphism for AWBMS 0022 was a little difficult to detect using SFR agarose; therefore, this genotype was confirmed on a polyacrylamide gel. For the M678 in the MIPs region, 22 primers were tested, of which 4 were polymorphic (EBmac701, EBmac635, EBmac679, Bmag0714B). EBmac701, EB mac635, and EBmac679 were mapped to the same location and Bmag714B was mapped to the other side of the M678 allele or gene in the MIPs locus. In this study, it was selected EBmac701 and Bmag714B as flanking markers for this locus.

Mapping of flanking markers

Flanking markers selected for each locus were genetically mapped in corresponding mapping populations (Table 2 and Figure 2). Using 188 homozygous families from an M422 x Steptoe population, the closest proximal marker for lpa1 was EBmac415, linked at 14.9 cM, on the distal side; Msu21 was the closest, at a distance of 9.6 cM. Orders of proximal and distal markers were decided based on the published barley 2006 consensus map in lpa1 locus (Marcel et al., 2007) at lpa1. M640 was previously mapped to the lpa2 area but its phenotype was obviously different from lpa2, indicating that it may be a different gene (Hu et al. manuscript submitted).

The mutant name of M640 was used to refer to the locus. Data from 2 populations were combined to select flanking markers for lpa2: polymorphism was identified between M640 and Steptoe as well as between M640 and Morex. The proximal marker, Bmag120, was linked at 13.7 cM; the distal marker, AWBMS0022, was linked at 11.8 cM. The orders of proximal and distal markers in the lpa2 area were decided based on the published map of barley Barque73 x CPI (GrainGenes, http://wheat.pw.us da.gov; Hearnden et al., 2007).

Mapping of the MIPS linked locus utilized a population of 41 families for the marker Bmag714B from M678 x Steptoe and 86 families for EBmac701 from both M678 x Steptoe and M678 x Morex populations. At 18.6 cM, EB mac701 was the closest proximal marker; Bmag714B, at 17.0 cM, was the closest distal marker. The proximal and distal orders of EBmac701 and Bmag714B were decided based on the same Barque73 x CPI map (GrainGenes, http:// wheat.pw.usda.gov; Hearnden et al., 2007).

SSR marker redundancy on chromosome 4H

Barley consensus maps from 2003 and 2005 differ in pri-

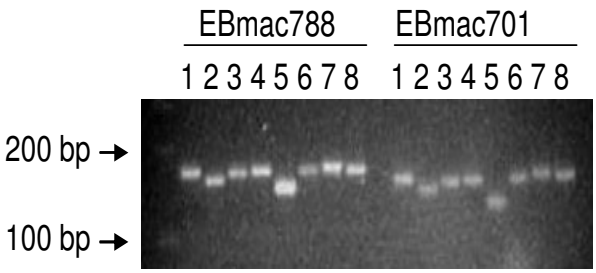

Figure 3. Comparison of amplification profiles of EBmac788 and EBmac701 using 8 DNA samples. Lane 1-8 represents the DNA of barley cvs. Harrington, Steptoe, Morex, CDC Alamo, Waxbar, Baronesse, barley germplasm Oregon Wolfe Barley Dominant, and barley cv. Azhul. PCR products were separated on 3% SFR agarose

mer content and marker position; however, both maps include EBmac701 and EBmac788 as discrete markers on chromosome 4H (GrainGenes, http://wheat. pw.usda. gov). Marker screening of the M678 in the MIPS region in this project initially included both markers. Results, however, indicate that EBmac788 is not a discrete marker but is a duplicate of EBmac701 in the opposite orientation. Comparison of primer sequences revealed that EBmac 701F is identical to EBmac788R (Table 3). Flanking primers in each pair shared a five-base sequence on one end, with unique sequences on opposite ends. Thus, the product size of these markers differs by 19 bp, although resultant genotypes are identical. To confirm the equivalence of these markers, amplification profiles were compared using DNA from barley cultivars Harrington, Steptoe, Morex, CDC Alamo, Waxbar, Baronesse, Azhul, and barley germplasm Oregon Wolfe Barley Dominant (Figure 3). Profiles were identical for the 2 markers.

To further clarify the marker order in this area, EBmac 635 and EBmac679 were mapped in the same mapping population of M678 x Steptoe. Results indicated that these 2 markers are proximal to EBmac701 at about 4.9 cM distance (Figure 2). Since these 2 markers were mapped between EBmac701 and EBmac788 (Ramsay et al., 2000), the correct mapping position for EBmac701/EB mac788 should correspond to EBmac788 in the published map.

DISCUSSION

Considerable interest has centered on development of lpa grain as a solution to dietary micronutrient deficiency and environmental pollution. Feed barley with lpa could provide a nutritious and cost-effective alternative to current feedstuffs (Li et al., 2001; Sugiura et al., 1998; Veum et al., 2002). Hence, lpa has become a significant criterion in development of new feed barley cultivars. Recently, barley lines with lpa genes have been released (Bregitzer et al., 2008) and there is strong evidence for upcoming popular utilization of the lpa mutant genes or alleles in feed barley development. Lower phytate and higher inorganic phosphorus content in lpa mutations not only provides more available P for non-ruminant animals but also reduces phytate contamination in ground water. The contribution of lpa lines to decreased water contamination may be more significant because a clean environment will extensively affect people's lives in a positive way. With better knowledge of the beneficial impacts of lpa genes or alleles, incorporation of those genes in cultivar development will be enhanced.

Efficient selection of this trait in barley breeding programs could be facilitated by flanking DNA markers. Marker assisted selection has been proved a rapid and effective approach to enhance the selections when genotypes of seedlings are required. Using markers is critical where more than one gene need to be pyramided because phenotype assessments for each individual will be difficult. While the data presented here coincided with mapping results in published literature (Larson et al., 1998; Roslinsky et al., 2007), development of flanking markers at 3 major lpa loci may enable more rapid progress in practical use of these mutations. Flanking DNA markers will assure the presence of a specific gene or allele in the individuals because false positive will be very rare.

The genetic explanation is very simple: double recombination between 2 flanking DNA markers occurs at a much lower frequency compared to recombination between one and a single marker. The markers identified in this study are PCR-based, and thus amenable to high-through put genotyping. For the majority of markers, polymorphism was unambiguously visualized in agarose gels, a further advantage for rapid laboratory analysis. Markers with difficult-to-detect polymorphism in the agarose gel, such as AWBMS0022, can be validated by sequencing based genotyping facility or use of polyacrylamide gels. Development of flanking DNA markers for Lpa loci in this report is only the start. Results provided a base for further fine-tuning the markers for tighter linkage and easier detection. Even though markers were developed in limited barley cultivars, testing of markers near the ones identified in this study in different barley lines should enable identification of useful ones.

In addition to lpa marker development, this study has improved the marker accuracy in the MIPS region of chromosome 4H. Global mapping efforts may generate multiple instances of marker redundancy, such as that detected for EBmac701 and EBmac788. The marker redundancy will result in misinterpretation of genetic maps because the redundant marker will show different mapping positions due to the different mapping populations used. The consensus map has to incorporate different maps because of the difficulty of re-testing all the markers in the same population. Inaccurate mapping positions or redundancy may be more problematic in gene fine mapping or map-based gene cloning. Therefore, it may be a good idea for the marker end-users to verify published marker positions and distances in the population of interest before using them. Publishing the confirmed

data will benefit the barley marker community.

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REFERENCES

Bregitzer PP, Raboy V, Obert DE, Windes J, Whitmore J (2008). Registration of 'Clearwater' low-phytate hulless spring barley. J. Plant Regist. 2: 1-4.

Chen PS, Toribara TY, Warner H (1956). Microdetermination of P. Anal. Chem. 28: 1756-1758.

See D, Kanazin V, Kephart K, Blake T (2002). Mapping Genes Controlling Variation in Barley Grain Protein Concentration. Crop Sci. 42: 680-685.

Erdman J (1981). Bioavailability of trace minerals from cereals and legumes. Cereal Chem. 58:21-26.

Graf E, Epson KL, Eaton JW (1987). Phytic acid: A natural antioxidant. J. Biol. Chem. 262: 11647-11650.

Hanakahi LA, Bartlet-Jones M, Chappell C, Pappin D, West SC (2000). Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. Cell 102: 721-729.

Hearnden P, Eckermann P, McMichael G, Hayden M, Eglinton J, Chalmers K (2007). A genetic map of 1000 SSR and DArT markers in a wide barley cross. Theor. Appl. Genet. 115: 383-391.

Jackson EW, Avant JB, Overturf KE, Bonman JM (2006). A quantitative assay of *Puccinia coronata* f. sp. *Avena* DNA in *Avena sativa*. Plant Dis. 90:629-636.

Larson SR, Raboy V (1999). Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: Correspondence with a low phytic acid mutation. Theor. Appl. Genet. 99:.27-36.

Larson SR, Rutger JN, Young KA, Raboy V (2000). Isolation and genetic mapping of a non-lethal rice (Oryza sativa L.) low phytic acid 1 mutation. Crop Sci. 40: 1397-1405.

Larson SR, Young KA, Cook A, Blake TK, Raboy V (1998). Linkage mapping of two mutations that reduce phytic acid content of barley grain. Theor. Appl. Genet. 97: 141-146.

Lemtiri-Chlieh F, MacRobbie EAC, Brearley CA (2000). Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells. Proc Nat Acad. Sci. USA. 97:8687-8692.

Li YC, Ledoux DR, Veum TL, Raboy V, Zyla K (2001). Low phytic acid barley improves performance, bone mineralization, and phosphorus retention in turkey poults. J. Appl. Poult. Res. 10: 178-185.

Loewus FA (1990). Inositol biosynthesis. In: Morŕe DJ, Boss WF, Loewus FA (eds) Inositol Metabolism in Plants. Wiley-Liss, New York pp.13-19.

Marcel TC, Varshney RK, Barbieri M, Jafary H , de Kock MJD, Graner A, Niks RE , de Kock MJ (2007). A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to *Puccinia hordei* and of defence gene homologues. Theor. Appl. Genet. 114: 487-500.

Menniti FS, Oliver KG, Putney JW, Shears SB (1993). Inositol phosphates and cell signaling: New views of InsP_5 and InsP_6. Trends Biochem. Sci. 18: 53-56.

Nilan RA, Sideris EG, Sander C, Konzak CF (1973). Azide – A potential mutagen. Mutation Res. 17:142-144.

Ockenden I, Falk DE, Lott JNA (1997). Stability of phytate in barley and beans during storage. J. Agric. Food Chem. 45: 1673-1677.

Raboy V (1997). Accumulation and storage of phosphate minerals. In: Larkins BA, Vasil IK (eds) Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Netherlands pp. 441-477.

Raboy V, Gerbasi PF, Young KA, Stoneberg SD, Pickett SG, Bauman AT, Murthy PP, Sheridan WF, Ertl DS (2000). Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol. 124: 355-368.

Ramsay L, Macaulay M, degli Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards KJ (2000).A simple sequence repeat-based linkage map of barley. Genetics 156:1997-2005.

Roslinsky V, Eckstein PE, Raboy V, Rossnagel BG, Scoles GJ (2007). Molecular marker development and linkage analysis in three low phytic acid barley (*Hordeum vulgare*) mutant lines. Mol. Breed 20: 323-330.

Sasakawa N, Sharif M, Hanley MR (1995). Metabolism and biological activities of inositol pentakisphsphate and inositol hexakisphosphate. Biochem. Pharmacol. 50: 137-146.

Sharpley AN, McDowell RW, Kleinman PJA (2001). Phosphorus loss from land to water: Integrating agricultural and environmental management. Plant and Soil 237: 287-307.

Shi J, Wang H, Hazebroek J, Ertl DS, Harp T (2005). The maize low-phytic acid 3 encodes a myo-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. Plant J. 42: 708-719.

Somers DJ, Isaac P, Edwards K (2004). A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 109: 1105-1114.

Sugiura SH, Raboy V, Young KA, Dong FM, Hardy RW (1998). Availability of phosphorus and trace elements in low-phytate varieties of barley and corn for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 170: 285-296.

Varshney RK, Marcel TC, Ramsay L, Russell J, Röder MS, Stein N, Waugh R, Langridge P, Niks RE, Graner A (2007). A high density barley microsatellite consensus map with 775 loci. Theor. Appl. Genet. 114: 1091-1103.

Veum TL, Ledoux DR, Bollinger DW, Raboy V, Cook A (2002). Low-phytic acid barley improves calcium and phosphorus utilization and growth performance in growing pigs. J. Anim. Sci. 80: 2663-2670.

Wilcox JR, Premachandra GS, Young KA, Raboy V (2000). Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci. 40: 1601-1605.

York JD, Odom AR, Murphy R, Ives EB, Wente SR (1999). A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science 285: 96-100.