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Characterization of lipoxygenase-1 null mutants in barley

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Abstract This study describes the discovery and characterization of lipoxygenase-1 (LOX-1) null mutants in barley. Six lines did not exhibit any significant LOX activity in the silenced seed extract. Immunological analysis showed that these lines lacked the authentic LOX-1 protein. Genetic analysis of the F_2 population revealed that this trait was governed by a single recessive gene located at the *LoxA* locus on chromosome 4H. The six LOX-1 null mutants shared similar features and the same unique polymorphism in a structural gene region, implying that these mutants might be derived from the same ancestral origin.

Keywords Barley · Lipoxygenase-1 · Null mutant · Gene mapping · Molecular marker

Introduction

Lipoxygenase (EC.1.13.11.12) (LOX) is a nonheme ferrus protein, which catalyzes the hydroperoxidation of polyunsaturated fatty acids with a 1.4-*cis*–*cis*-pentadiene structure. Seed LOXs are widely spread in many plant species. However, the physiological role of these LOXs is largely unclear (Santino et al. 2003).

In barley (*Hordeum vulgare* L.), two isozymes of seed LOX, LOX-1 and LOX-2 (Yabuuchi 1976; Baxter 1982; Doderer et al. 1992; Yang et al. 1993) were cloned and then mapped to the *LoxA* locus on chromosome 4H and the *LoxC* locus on chromosome 5H, respectively (van Mechelen et al. 1995, 1999). LOX-1 provides the predominant LOX activity in malt, and has a relatively low pI compared to LOX-2 (Yang et al. 1993; Yang and Schwarz 1995). LOX-1 and LOX-2 of barley catalyze the formation of 9-hydroperoxide (9-HPOD) and 13-hydroperoxide (13-HPOD), respectively (Yang et al. 1993).

From a brewer's point of view, LOX-1 is of great interest because 9-HPOD forms beer-deteriorating substances, such as *trans*-2-nonenal and trihydroxyoctadecenoic acid (THOD), during further reactions in the brewing process (Kobayashi et al. 1993; Kuroda et al. 2002, 2003). *Trans*-2-nonenal is known as a major component of the cardboard flavor in aged beer (Meilgaard 1975; Drost et al. 1990). THOD is known to have an adverse effect on the quality of beer in terms of foam stability and flavor (Bauer et al. 1977; Yabuuchi and Yamashita 1979; Kaneda et al. 2001; Kobayashi et al. 2002). In contrast to these functional characterizations of LOX-1 in the brewing process, little is known about its genetic variation in barley.

In soybean [*Glycine max* (L.) Merr.], LOX-null genes have been discovered (Hildebrand and Hymowitz 1982; Kitamura et al. 1983; Davies and Nielsen 1986), characterized, and successfully introduced into cultivars in order to improve the flavor of soybean products. Moreover, these LOX-null lines showed normal growth and agronomic performance (Narvel et al. 1998). If available, barley germplasm without LOX-1 activity may make it possible to breed barley varieties which have the potential to improve the flavor and foam stabilities of beer.

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The present study describes the discovery and characterization of the LOX-1 null mutants.

Materials and methods

Plant material

Barley germplasms from the Research Institute for Bioresources, Okayama University, Japan, were used to screen for the LOX-1 activity. A segregation analysis of a LOX-1-null trait was performed using the F_2 seeds ($n = 136$) derived from the cross between cultivar CDC Kendall and a LOX-1-null landrace line (code name SBOU2). The linkage analysis of the LOX-1 null gene was performed using DNAs of the F_2 plants ($n = 144$) derived from the same cross. Segregation of the LOX-1 type of F_2 plant was estimated by five independent LOX assays of F_3 seeds per line.

LOX assay

A single silenced seed was crushed with a hammer and suspended in 400 µl of cold extraction buffer (0.1 M sodium acetate, pH 5.5). The homogenate was incubated for 30 min on ice with occasional vortexing and then centrifuged at 3,000×*g* for 15 min. The supernatant was used as the crude enzyme solution. Protein concentration of the crude enzyme solution was determined using the protein assay reagent (Bio-Rad, Hercules, CA, USA), based on Bradford method with BSA as a standard. The crude enzyme was incubated at 24°C for 5 min in a cocktail (2 mM linoleic acid, 0.05% Tween 20, 0.1 M sodium acetate, pH 5.5). The reaction was stopped by adding an equal volume of BHT solution (0.8 mM 2-6-*t*-butyl-*p*-cresol in methanol), and allowed to stand for 30 min at -20°C. The reactant was centrifuged at 3,000×*g* for 20 min, and the supernatant was subjected to the hydroperoxide measurement according to Jiang et al. (1991). One unit of LOX activity was defined as the amount of enzyme, which produces 1 nmol of hydroperoxide (with cumene hydroperoxide as the standard) per min per 1 µg total protein extracted. Negative controls of the samples were prepared by inactivating individual crude enzymes by heat treatment at 100°C for 5 min.

Western blot analysis

The purified LOX-1 antigen produced in *E. coli* was prepared as previously described (Kuroda et al. 2002). The antibody was raised by injecting the purified LOX-1 antigen into a rabbit. This antibody recognized both LOX-1 and LOX-2. The total protein was extracted from a single silenced seed. The single seed was ground, suspended in 20 mM Tris–HCl (pH 7.5) containing Complete Mini (Roche diagnostic, GmbH), and left for 30 min on ice. The homogenate was centrifuged at 3,000×*g* for 15 min at 4°C. The protein concentration of the supernatant was measured using the Bio-Rad protein assay (Bio-Rad Laboratories) with bovine serum albumin as the standard. The extracted protein (3 µg) was separated by 10% (w/v) SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to an Immobilon-P membrane (Millipore Corporation, MA, USA) by electroblotting. The LOX protein was detected using the LOX-antibody at a dilution of 1:1000 (v/v). The alkaline phosphatase-conjugated anti-rabbit IgG was used at a dilution of 1:5000 (v/v). Detection was carried out in a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Isoelectric focusing (IEF) was performed using PhastGel pI 3–9 (Pharmacia Biotech AB, Uppsala, Sweden).

RFLP and CAPS analysis

Isolation of the genomic DNA, Southern blotting, and restriction fragment length polymorphism (RFLP) analysis were carried out as previously described (Kaneko et al. 1999). *JBC970* (Miyazaki et al. 2000) and *MWG2033* (Graner et al. 1991), which were previously mapped on the short arm of chromosome 4H, were used as the RFLP probes.

For the cleaved amplified polymorphic sequence (CAPS) analysis of the LOX-1 structural gene, two primers (5′-CCATCACGCAGGGCATCCTG-3′, 5′-GCGTTGATGAGCGTCTGCCG-3′) were designed based on a known LOX-1 cDNA sequence (van Mechelen et al. 1995). The amplification of the LOX-1 gene fragment was performed using the Expand High Fidelity system (Roche diagnostic, GmbH). The thermocycling conditions were (94°C for 1 min, 65°C for 2 min, 72°C for 3 min) 30 times and 72°C for 7 min. By means of sequence analysis, a polymorphic sequence in the amplified region was found at the splicing donor site of the fifth intron of LOX-1 gene in SBOU2, which disrupts a restriction site of *Afa*I (N. Hirota et al. unpublished data). The polymorphism was detected by digesting the amplified DNA with *Afa*I (*Afa*I-CAPS). The amplified DNA fragment was confirmed to be the targeted part of the LOX-1 structural gene by partial sequencing.

The linkage relationships were calculated using the MAPMAKER/Version 3.0b program with an LOD threshold of 3.00 (Lander et al. 1987).

Results

Screening of landrace lines for LOX activity

The LOX activity in the silenced seed was investigated for 1,152 landrace lines stocked at the Research Institute for Bioresources, Okayama University. Six lines [code number SBOU1 (OUI001), SBOU2 (OUI003),

SBOU3 (OUJ095), SBOU4 (OUJ695), SBOU5 (OUN345) and SBOU6 (OUN347)] did not exhibit any significant LOX activity compared to each negative control (two-sample *t* test at the 5% probability level, average of eight samples) (Table 1). The seeds harvested in different years also did not show any significant activity (data not shown). All of the lines shared the following common features regardless of their geographic origin: covered kernel, six-rowed kernel, blue kernel, occidental type of rachis-brittleness (Takahashi 1955), 'A type' of *β*-amylase thermostability (Kaneko et al. 2001), etc. (Table 1).

The *Afa*I-CAPS analysis for the LOX-1 structural gene was carried out for these six lines as described in the Materials and methods section (Fig. 1). Two major bands (about 0.70 and 0.52 kb) were observed for SBOU1, SBOU2, SBOU3, SBOU4, SBOU5 and SBOU6 (Fig. 1; lanes 2–7, respectively), whereas three major bands (about 0.70, 0.30 and 0.22 kb) were observed for CDC Kendall (Fig. 1; lane 2).

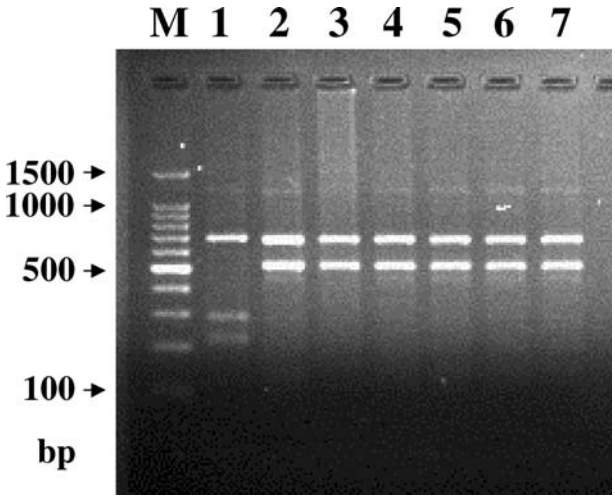

Fig. 1 The *Afa*I-CAPS analysis for the LOX-1 structural gene of LOX-1 null mutants. *Lane 1* CDC Kendall; *Lane 2* SBOU3; *Lane3* SBOU4; *Lane 4* SBOU1; *Lane 5* SBOU2; *Lane 6* SBOU5; *Lane 7* SBOU6; *Lane M* Molecular standards

Western analysis of seed LOX protein

In a preliminary experiment, the crude extract from the SBOU2 seed was added to that from the CDC Kendall seed (wild type regarding seed LOX-1). We could not detect any inhibitory activity for the LOX activity in the seed extract of SBOU2 (data not shown).

A Western analysis after SDS-PAGE (SDS-PAGE-Western) was performed for the total protein from the seeds of SBOU2. One major band at about 95 kDa was detected in the CDC Kendall seed, while two faint bands (approximately 95 and 57 kDa) were detected in SBOU2 (Fig. 2a). The Western analysis after IEF separation (IEF-Western) did not reveal a lower pI band around pI 4.8, but a high pI band around pI 6.5, indicating that the 95 kDa band of SBOU2 in the SDS-PAGE-Western analysis is derived from the LOX-2 protein based on a former report (Yang et al. 1993) (Fig. 2b).

In some cases, when a newly harvested seed was used in the LOX assay, the extracts from these LOX-1 mutant lines showed a very low but detectable level of total LOX activity. However, the authentic LOX-1 protein was not detected in those extracts by the IEF-Western analysis. This indicates that LOX-2 can occasionally provide a low activity in a silenced seed. Therefore, in that case, the LOX-1 phenotype of the line was confirmed using an additional IEF-Western analysis in the subsequent experiments.

The segregation of LOX-1 phenotype of silenced seeds

The segregation of the LOX-1 null phenotype was investigated using F_2 generation seeds ($n = 136$) derived from the cross between CDC Kendall and SBOU2. The segregation ratio of the wild type to the null phenotype was 104:32. The ratio was well fit to a 3:1 ratio

Table 1 Characteristics of LOX-1 null mutant lines

Characteristics	Germplasm					
	SBOU1	SBOU2	SBOU3	SBOU4	SBOU5	SBOU6
Seed LOX-1 activity	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
Covered or naked kernel	Covered	Covered	Covered	Covered	Covered	Covered
Kernel rows	6	6	6	6	6	6
Ear-awn type	Lax-long	Lax-long	Lax-long	Lax-long	Lax-long	Lax-long
Leaf-sheath hair	Absence	Absence	Absence	Absence	Absence	Absence
Heading time[b]	Mid-Apr.	Mid-Apr.	Mid-Apr.	Mid-Apr.	Late-Apr.	Mid-Apr.
Kernel color	Blue	Blue	Blue	Blue	Blue	Blue
Rachilla hair	Long	Long	Long	Long	Long	Long
Vernalization requirement[c]	HS	HS	HS	HS	S	S
Brittleness of rachis	Occidental	Occidental	Occidental	Occidental	Occidental	Occidental
β-amylase thermostability	Type A	Type A	Type A	Type A	Type A	Type A
Origin	India	India	Taiwan	Taiwan	Nepal	Nepal

[a]Not detectable
[b]Data in Okayama, Japan
[c]*HS* Highly spring, *S* Spring

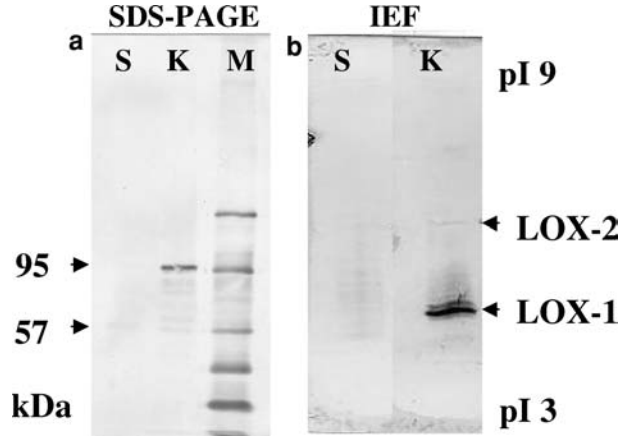

Fig. 2 Western blot analysis of the LOX-1 protein extracted from SBOU2 and CDC Kendall. **a** Western blot analysis after SDS-PAGE separation. **b** Western blot analysis after IEF separation. *Lane S* SBOU2; *Lane K* CDC Kendall; *Lane M* Molecular standard

($\chi^2 = 0.157$, $p = 0.69$), indicating that the LOX-1-null trait is governed by a single recessive gene in the Mendelian inheritance (Table 2).

Linkage analysis of the LOX-1 null gene

For the linkage analysis, the LOX-1 phenotype of F_2 plants was estimated based on the LOX assay data for F_3 seeds ($n = 5$) derived from individual F_2 plants ($n = 144$). The *Afa*I-CAPS analysis for the LOX-1 structural gene was carried out using their F_2 DNAs. All the plants with a homozygous SBOU2 type pattern in the *Afa*I-CAPS analysis exhibited the LOX-1 null phenotype and vice versa. The location on chromosome 4H was confirmed using a previously mapped RFLP probe, *JBC970* and *MWG2033*. Linkage analysis with MAPMAKER revealed that the LOX-1 null trait was assigned to a locus at a distance of 0, 4.1, and 10.5 cM from the LOX-1 structural gene, *JBC970* and *MWG2033*, respectively (Fig. 3).

Discussion

Barley LOX-1 has been studied from the point of beer quality, and shown to be involved in the synthesis of beer-deteriorating substances, such as *trans*-2-nonenal and THOD, through the formation of 9-HPOD (Kuroda et al. 2002, 2003). Therefore, it is important for the brewing industries to know the genetic diversity of the enzyme.

Table 2 Segregation analysis of LOX-1 null phenotype in F_2 population

Total	Wild type	Null type	χ^2(3:1)	*P*
136	104	32	0.157	0.69

In this study, we reported the discovery and genetic characterization of the LOX-1 null mutants of barley. In order to find the LOX-1 null barley lines, a silenced seed was used to screen the barley lines for LOX activity based on a report that the LOX-2 activity was detected only in germinating barley, while LOX-1 was present in silenced grain (Yang et al. 1993). Moreover, LOX-2 is less important in terms of the production of 9-HPOD (Yang et al. 1993; Yang and Schwarz 1995). As a result of the screening, we discovered six landrace lines (Table 1), which did not show any significant LOX activity and authentic LOX-1 protein in the silenced seeds (Table 1, Fig. 2). Furthermore, the extract did not inhibit the LOX activity of the barley seed (data not shown). These results clearly show that these six landrace lines are LOX-1 null mutants. Some LOX null mutants have been isolated and is well characterized in soybean (Hildebrand and Hymowitz 1982; Kitamura et al. 1983; Davies and Nielsen 1986). However, such an LOX null mutant has never been reported in barley. This is the first report of the LOX-1 null mutant lines in barley.

Interestingly, these six LOX-1 null lines shared similar characteristics (Table 1), such as covered kernel, six-rowed kernel, blue kernel, occidental type of rachis-brittleness (Takahashi 1955), "A type" of β-amylase thermostability (Kaneko et al. 2001). In addition, these lines shared the same unique *Afa*I-CAPS pattern (Fig. 1), which have never been observed in other lines tested so far. This close resemblance among these lines implies that these lines are derived from the same ancestral origin. This mutant probably originated in the Indo-Nepal region, and was introduced to Taiwan. We will continue a further investigation on the relationship among these lines.

A segregation analysis revealed a single recessive gene controlling the LOX-1 deficiency of SBOU2 (Table 2). The segregation of the phenotype was completely identical to that of the LOX-1 gene (Fig. 3). It was reported that the barley LOX-1 gene was located at the *LoxA* locus

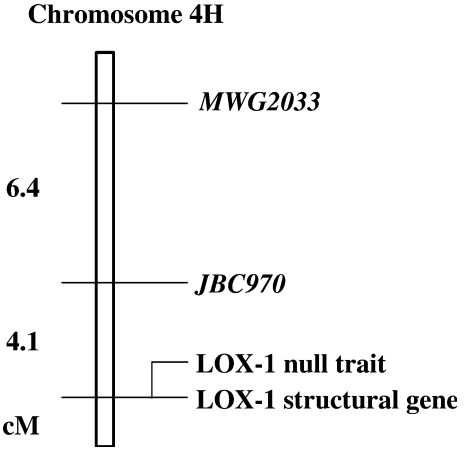

Fig. 3 A chromosome 4H segment showing molecular markers linking to the LOX-1 null trait

on the short arm of chromosome 4H (van Mechelen et al. 1999). Therefore, these results suggested that the deficiency of LOX-1 was due to a homozygous allele, *loxA*, which was recessive to the one (*LoxA*) required for the presence of the LOX-1 activity and protein (Fig. 3). This information will be effectively used in breeding programs to create advanced LOX-1 null malting barley varieties. Especially, the *Afa*I-CAPS analysis was simple and highly reproducible (Fig. 1). We have already determined the genomic and cDNA sequences of the LOX-1 gene in SBOU2 line. These DNA sequences suggest that the LOX-1 deficiency is caused by a single nucleotide mutation at the splicing donor site of its fifth intron, which creates the polymorphism at the *Afa*I site we used for the *Afa*I-CAPS analysis (N. Hirota et al. unpublished data). Therefore, this method can be successfully used for the direct selection of the LOX-null gene.

The beer made from a LOX-1 null population derived from a single cross between SBOU2 and a wild-type variety showed significant flavor and foam stabilities in a few brewing trials (N. Hiroata et al. unpublished data). Therefore, we believe that the LOX-1 null mutants discovered in this study would become one of the most powerful tools to improve the flavor and foam stabilities of beer without changing any beer production process. We are now introducing the LOX null trait into several advanced malting barley varieties in our breeding programs.

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References

Bauer C, Grosch W, Weiser H, Jugel H (1977) Enzymatic oxidation of linoleic acid: formation of bittertasting fatty acids. Z Lebensm Unters-Forsh 164:171–176

Baxter ED (1982) Lipoxydase in malting and mashing. J Inst Brew 88:390–396

Davies CS, Nielsen NC (1986) Genetic analysis of a null-allele for lipoxygenase-2 in soybean. Crop Sci 26:460–463

Doderer A, Kokkelink I, van Der Veen S, Valk B, Schram AW, Douma AC (1992) Purification and characterization of two lipoxygenase isozymes from germinating barley. Biochem Biophys Acta 1120:97–104

Drost BW, van den Berg R, Freijee FJM, van der Velde EG, Holleman SM (1990) Flavor stability. J Am Soc Brew Chem 48:124–131

Graner A, Jahoor A, Schondelmaier J, Siedler H, Pillen K, Fischback G, Wenzel G, Herrmann RG (1991) Construction of an RFLP map of barley. Theor Appl Genet 83:250–256

Hildebrand DF, Hymowitz T (1982) Inheritance of lipoxgenase-1 activity in soybean seeds. Crop Sci 22:851–853

Jiang ZY, Woollard ACS, Wollff SP (1991) Lipid hydroperoxide measurement of Fe^{2+} in the presence of Xylenol Orange. Comparison with the TBA assay and an iodometric method. LIPIDS 26:853–856

Kaneda H, Takashio M, Shinotsuka K, Okahata Y (2001) Adsorption and desorption of beer components from a lipid membrane related to sensory evaluation. J Biosci Bioeng 92:221–226

Kaneko T, Hirota N, Yokoi S, Kanatani R, Ito K (1999) Molecular marker for protein Z content in barley (*Hordeum vulgare* L.). Breed Sci 49:69–74

Kaneko T, Zhang WS, Ito K, Takeda K (2001) Worldwide distribution of β-amylase thermostability in barley. Euphytica 121:223–228

Kitamura K, Davies CS, Kaizuma N, Nielsen NC (1983) Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. Crop Sci 23:924–927

Kobayashi N, Kaneda H, Kano Y, Koshino S (1993) The production of linoleic acid hydroperoxides during mashing. J Ferment Bioeng 76:371–375

Kobayashi N, Segawa S, Umemoto S, Kuroda H, Kaneda H, Mitani Y, Watari J, Takashio M (2002) A new method for evaluating foam-damaging effect by fatty acid. J Am Soc Brew Chem 60:37–41

Kuroda H, Kobayashi N, Kaneda H, Watari J, Takashio M (2002) Characterization of factors that transform linoleic acid into di- and trihydroxyoctadecanoic acid in mash. J Biosci Bioeng 93:73–77

Kuroda H, Furusyo S, Maeba H, Takashio M (2003) Characterization of factors involved in the production of 2(E)-nonenal during mashing. Biosci Biotechnol Biochem 67:691–697

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage map of experimental and natural populations. Genomics 1:174–181

Meilgaard MC (1975) Flavor chemistry of beer: Part II: Flavor and threshold of 239 aroma volatiles. MBAA Tech Q 12:151–168

Miyazaki C, Osanai E, Saeki K, Hirota N, Ito K, Konishi T, Saito A (2000) A barley linkage map using an F2 population compared with a map based on female recombination-derived double haploid lines. Breed Sci 50:241–250

Narvel JM, Fehr WR, Welke GA (1998) Agromnomic and seed traits of soybean lines lacking seed lipoxygenases. Crop Sci 38:926–928

Santino A, De Paolis A, Gallo A, Quarta A, Casey R, Mita G (2003) Biochemical and molecular characterization of hazelnut (*Corylus avellana*) seed lipoxygenases. Eur J Biochem 270:4365–4375

Takahashi R (1955) The origin and evolution of cultivated barley. Adv Genet 7:227–266

Van Mechelen JR, Smit M, Douma AC, Rouster J, Cameron-Mills V, Heidekamp F, Valk BE (1995) Primary structure of a lipoxygenase from barley grain as deduced from its cDNA sequence. Biochem Biophys Acta 1254:221–225

Van Mechelen JR, Schuurink RC, Smits M, Graner A, Douma AC, Sedee NJA, Schmitt NF, Valk BE (1999) Molecular characterization of two lipoxygenases from barley. Plant Mol Biol 39:1283–1289

Yabuuchi S (1976) Occurrence of a new lipoxygenase in germinating barley embryo. Agric Biol Chem 40:1987–1992

Yabuuchi S, Yamashita H (1979) Gas chromatographic determination of trihydroxyoctadecanoic acid in beer. J Inst Brew 85:216–218

Yang G, Schwarz PB, Vick BA (1993) Purification and characterization of lipoxygenase isozymes in germinating barley. Cereal Chem 70:589–595

Yang G, Schwarz PB (1995) Activity of lipoxygenase isozymes during malting and mashing. J Am Brew Chem 53:45–49