

## Note

# The two-row malting barley cultivar ‘New Sachiho Golden’ with null lipoxygenase-1 improves flavor stability in beer and was developed by marker-assisted selection

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Lipoxygenase-1 (LOX-1) null ‘New Sachiho Golden’ is a two-row malting barley (*Hordeum vulgare* L.) cultivar released in 2015 that was developed at the Tochigi Prefectural Agricultural Experimental Station by backcross breeding using the high-yield leading cultivar ‘Sachiho Golden’ as a recurrent parent and the LOX-1 null mutant ‘Daikai LM1’ as a non-recurrent parent. To develop ‘New Sachiho Golden’ we used a simple LOX activity assay and marker-assisted selection. This is the first LOX-1 null malting barley cultivar in Japan that is resistant to barley yellow mosaic virus (types I–III). Agronomic characteristics and malting qualities of ‘New Sachiho Golden’ were similar to those of ‘Sachiho Golden’, except that ‘New Sachiho Golden’ had no LOX activity in ungerminated grains and had clearly lower LOX activity during malting than ‘Sachiho Golden’. The concentrations of a *trans*-2-nonenal (T2N) precursor in wort and beer made from ‘New Sachiho Golden’ were significantly lower than in those made from ‘Sachiho Golden’, both before and after storage.

**Key Words:** malting barley, backcross breeding, lipoxygenase-1, LOX-1 null, marker-assisted selection, Sachiho Golden.

## Introduction

Lipoxygenase-1 (LOX-1) in barley (*Hordeum vulgare* L.) grains and malt spoils beer flavor. LOX-1 oxidizes unsaturated fatty acids, such as linoleic acid, present in beer and generates *trans*-2-nonenal (T2N), which is the main cause of the cardboard off-flavor in beer closely associated with staling (Drost *et al.* 1990, Kuroda *et al.* 2002). Various ap-

proaches are used in malting and brewing to suppress the formation of this flavor and to preserve freshness (Ueda *et al.* 2001, 2004). Elimination of LOX-1 or decreasing its activity in malting barley reduces the amount of T2N in the resulting beer and increases its flavor stability (Hirota 2005).

The isozyme LOX-1 is present in both ungerminated and germinating barley grains, and is responsible for most LOX activity, whereas LOX-2 appears after germination (Mechelen *et al.* 1999, Yabuuchi 1976, Yang *et al.* 1993, Yang and Schwarz 1995). LOX-1 null barley is important to improve beer flavor and foam stability (Hirota *et al.* 2005, 2006); therefore, breeding of such barley has been proactively

Communicated by Hideyuki Funatsuki

Received June 24, 2016. Accepted November 30, 2016.

First Published Online in J-STAGE on March 17, 2017.

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promoted worldwide (Hoki *et al.* 2013). In Japan, although Sapporo Breweries Ltd. has released the new LOX-1 null cultivar ‘Satsuiku 2’ for Hokkaido Prefecture, no LOX-1 null cultivars suitable for the Honshu region have been developed.

Previously, we identified the LOX-1-deficient mutant line ‘Daikai LM1’, derived from the North American six-row malting cultivar ‘Karl’ treated with sodium azide, and developed a simple method to evaluate LOX-1 activity and a DNA marker for the barley gene encoding LOX-1 (Oozeki *et al.* 2007). However, ‘Daikai LM1’ has very late maturity and high susceptibility to barley yellow mosaic virus (BaYMV) and powdery mildew, which are undesirable traits in Japanese barley cultivars. Therefore, we used ‘Daikai LM1’ to introduce the LOX-1 null trait into the advanced malting barley cultivar ‘Sachiho Golden’ (Kato *et al.* 2006), which occupies more than 60% of the malting barley cropping area in Japan and about 90% of the area in Tochigi Prefecture (unpublished results of a survey by the Tochigi Prefectural Agricultural Administration in 2015; subject to non-disclosure). ‘Sachiho Golden’ is a high-yielding, high-quality malting cultivar and is the most widely used barley cultivar in Japan. Using the previously developed simple LOX evaluation method and DNA marker analysis (Oozeki *et al.* 2007), we developed ‘New Sachiho Golden’ by backcross breeding with ‘Sachiho Golden’ as the recurrent parent. This note describes our successful breeding process and the characteristics of ‘New Sachiho Golden’.

## Materials and Methods

### Breeding process

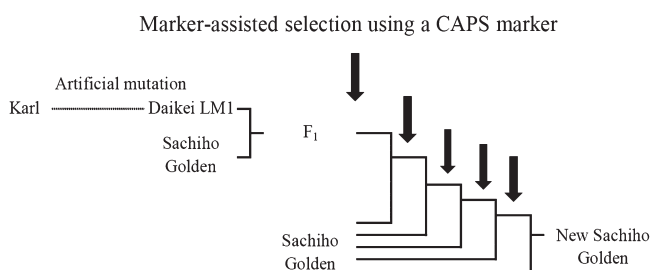
All crossings and selections were carried out at the Tochigi Prefectural Agricultural Experimental Station. The pedigree of ‘New Sachiho Golden’ (Fig. 1) began with the selection of ‘Daikai LM1’, derived from the six-row malting cultivar ‘Karl’ treated with sodium azide, as a LOX-1 null line. The cross of ‘Daikai LM1’ × ‘Sachiho Golden’ was performed in 2005. Five backcrosses and marker-assisted selection (MAS) using a cleaved amplified polymorphic sequence (CAPS) marker for the LOX-1 null gene of ‘Daikai LM1’ were performed in 2006 and 2007 to select heterozygous individual plants in each generation. BC<sub>5</sub>F<sub>1</sub> plants ( $n = 14$ ) were self-pollinated to obtain BC<sub>5</sub>F<sub>2</sub>, and single plants homozygous for the LOX-1 null trait were selected by using the simple evaluation method described by Oozeki

*et al.* (2007). BC<sub>5</sub>F<sub>2</sub> plants ( $n = 550$ ) were grown in the field in November 2007, and 122 plants were selected by observing the plant characteristics such as maturity stage, culm length, ear length, ear type, and LOX-1 activity measured by the simple evaluation method. From the BC<sub>5</sub>F<sub>3</sub> lines (a single pedigree of 122 selected plants), we selected the 58 best lines on the basis of earliness, culm length, plant type, and spike shape, and we investigated their grain quality and water sensitivity (reduction in germination rate in the presence of water excess to optimum). Grain quality was comprehensively determined by observation of size, weight, shape, uniformity, and color of the grain, based on the brewing barley survey criteria (National Agricultural Research Center 1986). Regarding variation in agricultural and malting characteristics in the 58 backcross lines (BC<sub>5</sub>F<sub>3</sub>), variation in water sensitivity was the largest (48.5%), and variation in maturity date was the smallest (1.2%). From among these 58 lines, 35 were used for malt production and malting quality analysis. In addition, we assayed these lines for LOX-1 deficiency by using the simple evaluation method. In 2008, one selected line was designated ‘Daikai HV36’. The line was renamed ‘Tochikei 348’ in 2009 and ‘Tochigi Nijo 45’ in 2011 after local adaptability tests, which were conducted in 2009 and 2010 at seven different research stations located respectively in Ibaraki, Gunma, Nagano, Aichi, Okayama, Yamaguchi and Saga Prefectures. At the same time, a test of specific characteristics of the breeding line and an analysis of malting quality were conducted in collaboration with the Brewers Association of Japan to determine its acceptability as a new production cultivar. In 2011–2014, performance tests for recommended cultivars were conducted at nine different stations located respectively in Gunma, Saitama, Tottori, Shimane, Okayama, Yamaguchi, Saga, Oita and Tochigi Prefectures. In 2015, ‘Tochigi Nijo 45’ was released as ‘New Sachiho Golden’, and an application for cultivar registration was submitted to the Ministry of Agriculture, Forestry and Fisheries of Japan. Commercial-scale malting and brewing trials are now underway.

### Lipoxygenase activity

LOX can occur as either LOX-1 or LOX-2, but only LOX-1 has been detected in ungerminated barley grains (Mechelen *et al.* 1999, Yabuuchi 1976, Yang *et al.* 1993, Yang and Schwarz 1995). The method of detection we used is not specific to LOX-1, so to take into account the potential presence of LOX-2 during germination, we refer to LOX enzyme activity in barley malt and during malting as LOX activity or total LOX activity.

LOX activity in individuals from early and middle generations (F<sub>2</sub>–F<sub>3</sub>) and during screening for a LOX-null mutant line was analyzed using the simple evaluation method (Oozeki *et al.* 2007), which allows the presence or absence of LOX to be identified. LOX activity in advanced generations and cultivars was analyzed using the standard method modified from Lulai and Baker (1976).



**Fig. 1.** Pedigree of ‘New Sachiho Golden’.

**Table 1.** Lipoxygenase-1 activity in ungerminated grains of the new malting barley cultivar 'New Sachiho Golden'

Cultivar name	Lipoxygenase-1 activity (units/g) <sup>a</sup>			
	2012	2013	2014	Average
New Sachiho Golden	0 <sup>b</sup>	0	0	0
Sachiho Golden	80	78	62	74
Sukai Golden	78	62	62	68
Asuka Golden	54	57	72	61

<sup>a</sup> One unit of lipoxygenase activity is defined as the increase in absorbance at 234 nm per minute at 25°C, per gram of barley flour.

<sup>b</sup> Activity was below the detection limit.

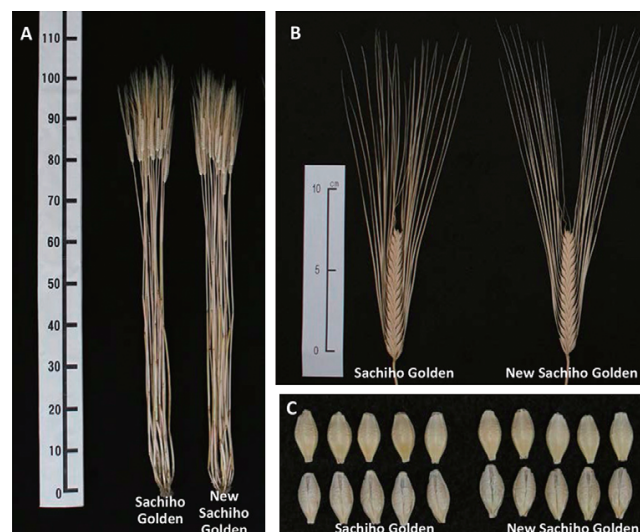
### Agronomic characteristics

The field agronomic characteristics of 'New Sachiho Golden' and 'Sachiho Golden' were compared using a total of 50 datasets obtained over 7 years at 14 locations.

We tested resistance to barley yellow mosaic virus (BaYMV), powdery mildew and scab, pre-harvest sprouting, and hull cracking (see **Table 3** for methods). The BaYMV strains (types I–V) have been classified on the basis of pathogenicity to various barley cultivars in Japan (Kashiwazaki *et al.* 1989, Sotome *et al.* 2010, Sotome 2012). To evaluate resistance to type III, we also used PCR analysis to examine polymorphism of the DNA marker k09554-*Ava*I, which is linked to *rym3* (Haruyama *et al.* 2012). The primer design was based on k09554 (Sato *et al.* 2009).

### Malting qualities

Malt samples were prepared using an automatic micro-malting system (Phoenix Systems, Adelaide, SA, Australia) according to the method for small-scale malting and brewing for quality analysis (Tochigi Prefectural Agricultural Experiment Station 1998). General malt characteristics were analyzed according to the methods of the European Brewery Convention (1987); these included ex-steep moisture, malt yield, wort clarity, color, extract, total and soluble nitrogen, Kolbach index (KI), diastatic power, viscosity and wort  $\beta$ -glucan content.

**Fig. 2.** Photographs of (A) plants, (B) spikes and (C) grains of I. 'Sachiho Golden' (recurrent parent) and II. 'New Sachiho Golden'.

## Results

The LOX-1 activity in ungerminated grains of 'Sachiho Golden' was 74 units/g, whereas that of 'New Sachiho Golden' was below the detection limit (**Table 1**).

The field agronomic characteristics of 'New Sachiho Golden' and 'Sachiho Golden' were analyzed by three-way ANOVA (**Table 2**). The differences among test locations were significant for all characteristics of both cultivars. The differences among years were significant for maturity date and 1000-grain weight. No interactions of test location by cultivar or of cultivar by year were found. Plant, spike, and grain appearances and the agronomic characteristics of 'New Sachiho Golden' were very similar to those of 'Sachiho Golden' (**Fig. 2**, **Table 2**).

'New Sachiho Golden' was highly resistant to BaYMV types I–III, but not to types IV and V (**Table 3**). 'New Sachiho Golden' is likely to have retained the resistance gene *rym3* from 'Sachiho Golden' on the basis of the results of a BaYMV field test and analysis of PCR products using

**Table 2.** Agronomic characteristics of the new malting barley cultivar 'New Sachiho Golden'

Cultivar <sup>a</sup>	Maturity date <sup>b</sup>	Culm length (cm)	Spike length (cm)	Number of spikes (/m <sup>2</sup> )	Plump grain yield (kg/a)	Volume weight (g/L)	1000-grain weight (g)
New Sachiho Golden	147.7	85	6.4	608	46.1	734	47.2
Sachiho Golden	147.1	86	6.5	610	46.1	735	47.5
Results of ANOVA							
Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Region	** <sup>c</sup>	**	**	**	**	**	**
Year	**	n.s.	n.s.	n.s.	n.s.	n.s.	**
Interactions <sup>d</sup>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

<sup>a</sup> The cultivars were tested simultaneously; a total of 50 datasets were obtained over 7 years in 14 regions.

<sup>b</sup> Days to maturity from the beginning of the year.

<sup>c</sup> Significantly different at \*\*1% level and \*5% level; n.s., not significant.

<sup>d</sup> All interactions between and among factors were not significant.

**Table 3.** Resistance to diseases, pre-harvest sprouting, and hull cracking in ‘New Sachiho Golden’ and four other cultivars of malting barley

Cultivar	BaYMV <sup>a</sup>					resistance gene	Powdery mildew <sup>b</sup>	Scab <sup>c</sup>	Pre-harvest sprouting <sup>d</sup>	Occurrence of hull-cracked grain <sup>e</sup> (%)
	I	II	III	IV	V					
New Sachiho Golden	RR <sup>f</sup>	RR	RR	M	M	<i>rym3</i>	RR	R	M	23
Sachiho Golden	RR	RR	RR	MS	M	<i>rym3</i>	RR	R	M	25
Mikamo Golden	RR	RR	S	RR	RR	<i>rym5</i>	M	R	MS	9
Sukai Golden	RR	RR	RR	RR	RR	<i>rym3+5</i>	RR	R	R	24
Asuka Golden	RR	RR	RR	RR	RR	<i>rym3+5</i>	RR	R	MS	8

<sup>a</sup> Assessed on the basis of the rate of diseased plants in the BaYMV test field, resistance was classified into seven categories (see footnote *f*).

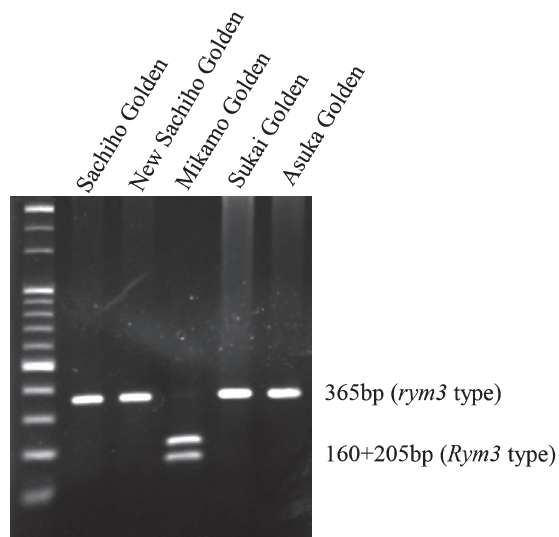
<sup>b</sup> Determined on the basis of the degree of lesion spots due to natural pathogenesis in spring sowing condition, resistance was classified into seven categories (see footnote *f*).

<sup>c</sup> Tested in the Nagano Agricultural Experiment Station. Resistance to scab was estimated according to Yoshida *et al.* (2003). Resistance was classified into seven categories (see footnote *f*).

<sup>d</sup> Ten spikes were harvested at maturity, steeped for 1 day in water at 17°C, and incubated for 6 days at 17°C and 100% humidity. The germination rate of grain from each spike was determined visually and the average value of 10 spikes was calculated. Resistance was classified into seven categories (see footnote *f*).

<sup>e</sup> Estimated according to Yoshikawa *et al.* (1995).

<sup>f</sup> RR: highly resistant; R: resistant; MR: moderately resistant; M: moderate; MS: moderately susceptible; S: susceptible; SS: highly susceptible.



**Fig. 3.** Analysis of the PCR products using the DNA marker k09554-*Ava*I for the gene for barley yellow mosaic virus resistance, *rym3*.

the DNA marker k09554-*Ava*I (Table 3, Fig. 3). In comparison with ‘Sachiho Golden’, ‘New Sachiho Golden’ seemed to be less susceptible to type IV (Table 3); the difference in resistance between cultivars depended on environmental factors. Both cultivars had the same levels of resistance to other pathogens tested.

The malting quality properties of ‘New Sachiho Golden’ and ‘Sachiho Golden’ were also analyzed by three-way ANOVA (Table 4). The differences among test locations were significant for all characteristics of both cultivars. The differences among years were significant for water sensitivity, apparent final attenuation, and wort viscosity. No significant interactions between test location and cultivar or cultivar and year were found. These data indicate that the excellent malting quality of ‘Sachiho Golden’ is retained in ‘New Sachiho Golden’.

We investigated malting quality under low, standard and high (extended immersion) steeping regimes (Table 5). Malting quality changed with increasing immersion time

**Table 4.** Malting quality properties of ‘New Sachiho Golden’ versus those of ‘Sachiho Golden’

Cultivar <sup>a</sup>	Water sensitivity <sup>b</sup>	Malt extract (%)	Protein content of malt (%)	Soluble nitrogen content in wort (%)	Kolbach index <sup>c</sup> (%)	Diastatic power (WK/TN) <sup>d</sup>	Apparent final attenuation (%)	Wort β-glucan (mg/L)	Wort viscosity (mPa·s)
New Sachiho Golden	6	84.6	9.8	0.74	46.7	226	83.7	134	1.55
Sachiho Golden	6	84.7	9.8	0.74	46.8	218	82.8	122	1.56
Results of ANOVA									
Cultivar	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Region	** <sup>e</sup>	**	**	**	**	**	**	**	n.s.
Year	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	**
Interactions <sup>f</sup>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

<sup>a</sup> The cultivars were tested simultaneously; a total of 31 data sets were obtained over 5 years in 11 regions.

<sup>b</sup> Water sensitivity was determined following the procedure of Tochigi Prefectural Agricultural Experiment Station (1998).

<sup>c</sup> Kolbach index is the proportion of soluble nitrogen per malt total nitrogen.

<sup>d</sup> WK is an indicator of the saccharification ability; TN, malt total nitrogen.

<sup>e</sup> Significantly different at \*\*1% level and \*5% level; n.s., not significant.

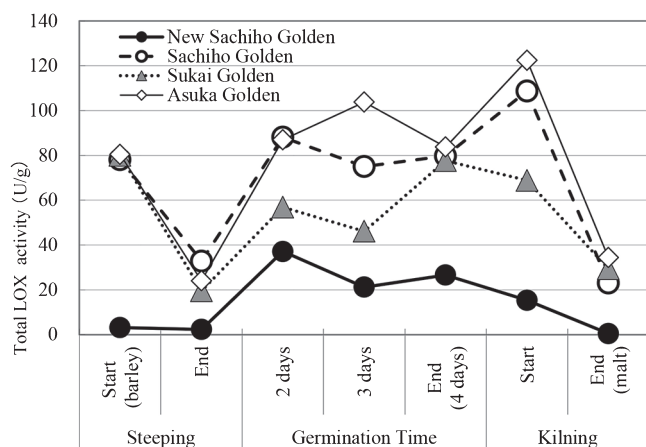
<sup>f</sup> All interactions between and among factors were not significant.

**Table 5.** Effects of steeping regime on malting quality of 'New Sachiho Golden' and three other cultivars of malting barley

Cultivar	Protein content of grain	Steeping time	Steep moisture	Malt yield	Color	Malt extract	Protein content of malt	Soluble nitrogen content in wort	Kolbach index	Diastatic power	Examination marks for quality test of malt	Wort $\beta$ -glucan	Viscosity
	%	h	%	%	$^{\circ}$ EBC <sup>a</sup>	%	%	%	–	WK <sup>b</sup>		mg/L	mPa s
New Sachiho Golden	10.7	42.5	Low	93.9	2.4	84.3	10.5	0.68	40.4	295	81.0	128	1.62
		48.5	Standard	92.0	2.8	83.9	10.6	0.73	42.8	310	84.7	33	1.53
		58.4	High	91.1	2.7	83.7	10.4	0.74	44.1	314	84.1	16	1.53
Sachiho Golden	10.3	43.0	Low	93.5	2.2	84.9	10.2	0.68	41.4	307	83.6	112	1.64
		48.5	Standard	91.9	2.4	84.6	10.0	0.70	43.9	306	86.4	37	1.60
		58.0	High	90.8	2.4	84.5	10.0	0.71	44.4	321	88.5	21	1.55
Sukai Golden	11.0	38.2	Low	93.6	2.4	83.3	11.1	0.76	42.8	307	80.3	55	1.54
		49.4	Standard	91.4	2.5	83.2	10.9	0.80	45.6	315	81.6	17	1.49
		53.9	High	90.7	2.7	83.3	11.0	0.82	46.6	326	79.6	16	1.49
Asuka Golden	10.7	45.6	Low	94.4	2.2	83.9	10.5	0.66	39.3	329	80.5	40	1.53
		51.5	Standard	93.7	2.3	84.0	10.7	0.68	40.0	331	83.9	21	1.53
		60.3	High	92.8	2.3	83.4	10.6	0.69	40.5	338	82.4	14	1.52

<sup>a</sup> Value of the congress wort at a fixed time after filtration was analyzed using the EBC glass disk.

<sup>b</sup> WK is an indicator of saccharification ability.



**Fig. 4.** Total LOX activity in 'New Sachiho Golden' and three other malting barley cultivars during micro-malting by Asahi Breweries, Ltd. in 2015.

**Table 6.** Concentrations of the trans-2-nonenal (T2N) precursor and T2N in wort and beer made from 'New Sachiho Golden' and three other malting barley cultivars tested by Asahi Breweries, Ltd. in 2015

Cultivar name	Precursor of T2N		T2N <sup>a</sup>	
	wort	beer	wort	beer
New Sachiho Golden	2.6 a <sup>b</sup>	1.4 a	0.8 a	0.2 a
Sachiho Golden	9.6 b	3.5 a	2.5 b	0.7 b
Sukai Golden	10.8 b	3.9 a	2.5 b	0.8 b
Asuka Golden	8.8 b	3.8 a	1.9 ab	0.7 b

<sup>a</sup> Measured after storage at 37°C for 1 week.

<sup>b</sup> Values with the same letter within a column are not significantly different (Tukey,  $P < 0.1$ ).

similarly in all cultivars tested when compared to 'Sachiho Golden' as the control variety and 'Sukai Golden' and 'Asuka Golden' as check varieties.

The total LOX activity of 'New Sachiho Golden' during

micro-malting was clearly lower than that of the other cultivars tested (Fig. 4). The T2N precursor concentrations in wort and fresh beer made from 'New Sachiho Golden' immediately after micro-scale brewing and the T2N concentration in wort and beer stored at 37°C for 1 week (equivalent to storage at 25°C for 1 month) were clearly lower than those of beer made from the other cultivars (Table 6). These results confirm the expected effect of the LOX-1 null trait on T2N concentration.

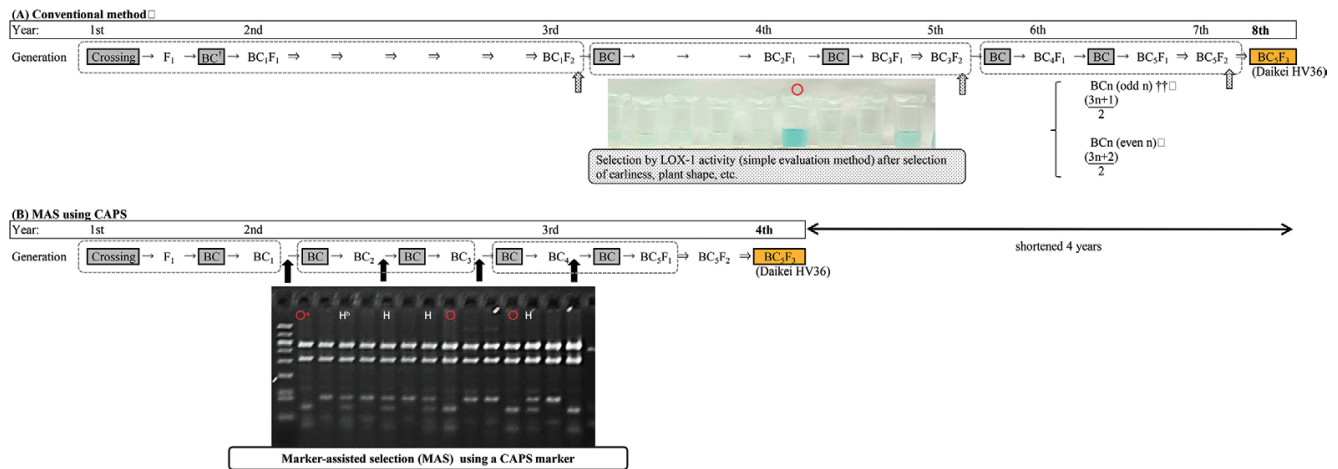
## Discussion

Breeding of malting barley in Japan usually takes about 14 years for pedigree selection and 2 years for malting and brewing testing at the commercial scale (40–80 t); that is, overall, at least 16 years. Therefore, shortening the breeding term and improving the breeding method are desirable (Oozeki *et al.* 2013). Combining the use of selection based on both the simple LOX evaluation method and the DNA marker reduced the time needed to 10 years.

LOX-1 null trait could be introduced into the leading cultivar 'Sachiho Golden' by using MAS and a simple LOX activity evaluation method. In our procedure, we could obtain BC<sub>5</sub>F<sub>3</sub> lines for small-scale performance test and malting quality selection approximately 4 years sooner than would be expected by following a conventional breeding process (Fig. 5). We must cultivate three times to backcross twice in the conventional method, but cultivated two times in our procedure. It can be said it was 1.5 times the efficiency of breeding.

We believe this outcome would not have been possible with the use of only one selection method. To further improve breeding efficiency, the use of multiple DNA markers might be advantageous because it would ensure that the desirable traits of the recurrent parent are preserved.

Efforts to breed LOX-1 null barley have been underway



**Fig. 5.** Comparison of breeding methods by (A) conventional LOX-1 activity screening and (B) *lox-1* identification using marker-assisted selection (MAS) as used in this study to breed ‘New Sachiho Golden’. † BC: backcrossing. †† Required number of generations. <sup>a</sup> ○: homozygous for the *lox-1*. <sup>b</sup> H: heterozygous for the *lox-1*. <sup>c</sup> Dashed frames indicate the smallest unit of repetitive tasks. <sup>d</sup> The black arrows indicate selection from MAS and the gray arrows indicate selection by LOX-1 activity screening after selection for earliness, plant shape and other attributes.

in Denmark (with the release of ‘Charmay’, ‘Cha Cha’ and ‘Cherrio’), Canada (‘CDC PolarStar’), and Australia (‘SouthernStar’) (Gleadell 2015, Hoki *et al.* 2013). ‘CDC PolarStar’ has become a major cultivar (based on area) in Canada. Although the use of LOX-1 null barley cultivars is expanding in other countries, in Japan, LOX-1 null barley is currently cultivated only in Hokkaido Prefecture. Breeding of LOX-1 null barley has been strongly demanded by Japanese breweries, so we consider the introduction of this trait to be important for the breeding of domestic malting barley.

In germinating barley grains, the isozymes LOX-1 and LOX-2 have been identified and characterized. Elimination or reduction of both isozymes would further improve malting quality. Following the method for the development of ‘New Sachiho Golden’, we intend to develop a new cultivar with reduced total LOX activity. We hope that the release of ‘New Sachiho Golden’ will lead to quality improvement and expand the production of domestic malting barley in Japan.

## Acknowledgments

This research was financially supported by grants to the designated research station system from the Ministry of Agriculture, Forestry, and Fisheries and National Projects (23010 and 26105C) of the Agriculture, Forestry and Fisheries Research Council.

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