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K. Tanno · S. Taketa · K. Takeda · T. Komatsuda

A DNA marker closely linked to the *vrs1* locus (row-type gene) indicates multiple origins of six-rowed cultivated barley (*Hordeum vulgare* L.)

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Abstract The origin of six-rowed cultivated barley was studied using a DNA marker cMWG699 closely linked to the *vrs1* locus. Restriction patterns of the PCR-amplified product of the cMWG699 locus were examined in 280 cultivated (*Hordeum vulgare* ssp. *vulgare*) and 183 wild (*H. vulgare* ssp. *spontaneum*) barleys. Nucleotide sequences of the PCR products were also examined in selected accessions. Six-rowed cultivated barleys were divided into two distinct groups, types I and II. Type I six-rowed cultivated barley was distributed widely while type II six-rowed cultivated barley was found only in the Mediterranean region. The type I sequence was also found in a wild barley accession from Turkmenistan whereas the type II sequence was also found in a two-rowed cultivated barley from North Africa and a wild barley from Morocco. These results suggested that the six-rowed type I and II barleys were derived from two-rowed type I and II barleys, respectively, by independent mutations at the *vrs1* locus.

Keywords Barley · Six-rowed · Origin · The*vrs1* locus · Nucleotide sequence

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K. Tanno · T. Komatsuda
National Institute of Agrobiological Resources, Kannondai,
Tsukuba 305-8602, Japan

K. Tanno
Institute of Agriculture and Forestry, University of Tsukuba,
Tsukuba, 305-8572, Japan

S. Taketa
Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa
761-0795, Japan

K. Takeda
Research Institute for Bioresources, Okayama University, Chuo,
Kurashiki 710-0046, Japan

Present address:
K. Tanno (✉), Research Institute for Bioresources,
Okayama University, Chuo, Kurashiki 710-0046, Japan
e-mail: tannok@rib.okayama-u.ac.jp
Tel.: +81-86-424-1661, Fax: +81-86-434-1249)

Introduction

Barley (*Hordeum vulgare*) is one of the main crops in world cereal production and is also one of the earliest cultivated crops of the world along with emmer wheat (*Triticum turgidum* ssp. *dicoccon*), einkorn wheat (*Triticum monococcum*), lentil (*Lens culinaris*) and pea (*Pisum sativum*) (Zohary and Hopf 1993). Unlike bread wheat (*Triticum aestivum*) that experienced polyploidization events, the domestication of barley is assumed to have taken place from wild barley, subspecies *spontaneum*, in the Near East (Harlan and Zohary 1966). Population genetic studies based on biochemical markers (Nevo et al. 1986) and chloroplast DNA variation (Neale et al. 1988) demonstrated much less variation of cultivated barley as compared to wild barley, suggesting that cultivated barley had originated from a small number of wild barley plants. However, the sites of the domestication events in barley remain unclear.

Row type of the spikelet is one of the key characters for the elucidation of the origin of cultivated barley. Wild barley is only two-rowed while cultivated barley has two- and six-rowed spikelets, and six-rowed cultivated barley is more widely distributed than two-rowed cultivated barley. Six-rowed barley was the main cereal even in ancient Mesopotamia (the 4th millennium BC). De Candolle (1882) was the first to study the origin of barley. He mentioned two possibilities for the ancestors of cultivated barley; '*Hordeum spontaneum*', or six-rowed wild barley which once existed but became extinct within historic time. Later, a number of researchers argued whether the progenitor of cultivated barley was two- or six-rowed, maintaining several opinions: the "two-rowed progenitor hypothesis" (Helbaek 1959; Bakhteyev 1963; Harlan 1968), the "six-rowed progenitor hypothesis" (Schiemann 1932; Åberg 1940) and the mixed "di-phyletic hypothesis" (Freisleben 1940; Takahashi 1955). Today, the two-rowed hypothesis has been generally accepted because: (1) extant wild barley has two-rowed spikelets while the six-rowed brittle form sometimes found in Tibet and Israel, and designated as the wild six-rowed form, viz. '*H. agriocrithon*', is regarded as a hybridization product of two-rowed wild barley with six-rowed cultivated barley (Zohary 1963; Bothmer et al. 1995); (2) before the cultivation of barley, the two-rowed brittle barley such as the ssp. *spontaneum* form was utilized as food (Harlan 1968); and (3) the two- and six-rowed spikelets are controlled at the *vrs1* (formerly *v*) locus in chromosome 2H (Griffee 1925) (the new gene symbol follows Franckowiak et al. 1997), and the two-rowed type is dominant while the six-rowed type is recessive (Ubisch 1916). Mutation at this locus by irradiation caused the change from the two- to six-rowed type (Nybom 1954; see Lundqvist et al. 1997) but the reciprocal is unknown. If indeed six-rowed barley evolved from the two-rowed type, then the present six-rowed barley seems to have originated from a limited number of two-rowed barley. Clarification of the sites of origin of six-rowed barley would provide important information on the crop history and will also be useful for future barley breeding.

Since genes at the *vrs1* locus have not been cloned yet, this study used the cMWG699 marker, which was linked to the *vrs1* locus with a map distance of 0.1 cM (Graner et al. 1991; Komatsuda et al. 1999). In a previous study (Tanno et al. 1999), restriction analysis of the amplified DNA of this marker showed three haplotypes in barley cultivars, which were named A, D and K; and analysis of their DNA sequence suggested that six-rowed barleys of types A and D had differentiated from two-rowed barleys of types A and D, respectively. However, this study included a small number of cultivated barleys, so that the conclusion was not solid, and the data did not tell us anything about the domestication of barley because wild barley was not included. In the present study, the geographical distribution of the three haplotypes was investigated using a larger number of cultivated and wild barley accessions, and their nucleotide sequence was analyzed to elucidate the origin of the six-rowed cultivated barley.

Materials and methods

Plant materials

This study included 280 cultivated and 183 wild (ssp. *spontaneum*) barley accessions (Table 1). The cultivated barleys were mostly landraces; 76 were two-rowed and 204 were six-rowed. Materials from the new continents and two-rowed barleys from the Far East were not included, since such materials are considered to have been introduced recently. Some brittle rachis six-rowed forms '*H. agriocrithon*' are found in Tibet and Israel, but they are probably hybridization products between ssp. *spontaneum* and six-rowed cultivated barley (Zohary 1963; Bothmer et al. 1995). For this reason, the *H. agriocrithon* form was not included in this study. All the barley germplasms used are collections of the Barley Germplasm Center, Research Institute for Bioresources, Okayama University, Kurashiki, Japan, except for 12 Israeli, 6 Jordanian, 3 Turkish and 1 Syrian accessions of ssp. *spontaneum* which were obtained from USDA. The row type of spike was determined based on the fertility of the lateral spikelets.

DNA amplification and restriction analysis

Total DNA was extracted from young leaves as described by Komatsuda et al. (1998). PCR amplification of cMWG699 and restriction analysis with *Taq*I (Nippongene) were performed following the methods described by Komatsuda et al. (1998). For simplification of gel electrophoresis (3% MetaPhor, FMC, Rockland, Maine), the internal primers cMWG699 T7–3 and T3–3 (see Tanno et al. 1999), instead of external primers cMWG699 T7–2 and T3–2, were used to amplify the intron part.

DNA sequencing

DNA amplification was performed by PCR using the cMWG699 T7–2 and T3–2 primers (Komatsuda et al. 1998). Amplified DNA

Table 1 Barley germplasms used in this study. For information about the collection history of each accession, contact Tanno or Komatsuda

Region	Abbreviation	Cultivated (280)		Wild (183)	Accession
		2-Rowed	6-Rowed	2-Rowed	
North and Central Europe	NCEU	28	10		Czechoslovakia, Denmark, Finland, Germany, Hungary, Poland, Romania, Sweden, Switzerland, United Kingdom
South Europe	SEU	7	16		Bulgaria, France, Italy, Spain, Yugoslavia
North Africa	NA	3	10	9	Algeria, Egypt, Libya, Morocco, Tunisia, an unknown country
Ethiopian	ETP	9	28		Ethiopia
Middle East	ME	29	21	123	Armenia, Azerbaijan, Cyprus, Georgia, Iran, Iraq, Israel, Jordan, Syria, Turkey
South and Central Asia	SCA		10	51	Afghanistan, India, Pakistan, Tajikistan, Turkmenistan
Himalayan	HL		32		Nepal, Tibet[a]
Far East	FE		77		China[a], Japan, North Korea, South Korea
Total		76	204	183	

[a] Tibet is dealt with separately from China in this study

was cloned into the pCRII vector by using the TA cloning kit (Invitrogen). Nucleotide sequences were determined with Fluorescence Cycle Sequencing Kits (Applied Biosystems) and a 373 A automated DNA sequencer (Perkin-Elmer). Three independent clones were analyzed for each accession to eliminate PCR-amplification artifacts. The DNA sequences were aligned manually.

Results

Geographical distribution of cMWG699/*Taq*I polymorphism

The PCR amplification generated a single DNA fragment of about 490 bp in all the barley accessions. The *Taq*I digestion of the PCR products showed three haplotypes A, K and D in the materials (Fig. 1). The three haplotypes corresponded to the A, K and D types detected in a previous study (Tanno et al. 1999), and no other type was detected in this study.

Figure 2 shows the geographical distribution of the three haplotypes. In ssp. *spontaneum* (Fig. 2a), type A was predominant (172 out of 183) and was distributed in a large area of the Middle East, and South and Central Asia. In contrast, only four accessions were of type K which were obtained from Iran (three accessions) and Israel (one accession). Type D was found only in North Africa. Seven accessions out of nine were of type D and they were from Morocco (the other two were of type A from Libya). We picked up a number of Moroccan ssp. *spontaneum* in this region since some reports suggest that Morocco is one of the domestication centers for cultivated barley (see Discussion).

In two-rowed cultivated barley (Fig. 2b), 38 accessions were of type A, 36 were of type K and two were of type D. The proportion of type K was high in North and Central Europe (85%) and Southern Europe (86%), but low in the Middle East (17%), and none in Ethiopia (0%). In North Africa, only three cultivars were analyzed because two-rowed cultivated barley is rare in this region (Yasuda et al. 1993). They were of types A, K and D.

In six-rowed cultivated barley (Fig. 2c), type A was prevalent all over the regions examined and was predominant in number. In the 204 accessions analyzed, type D was found in only seven accessions and all of them were from Southern Europe (Spain, France, Italy, Yugoslavia and Bulgaria). Type K was not found in six-rowed cultivated barley.

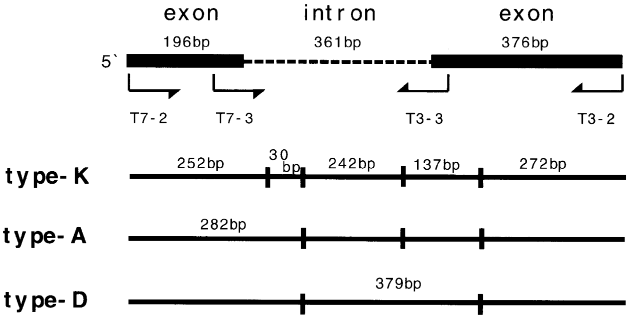
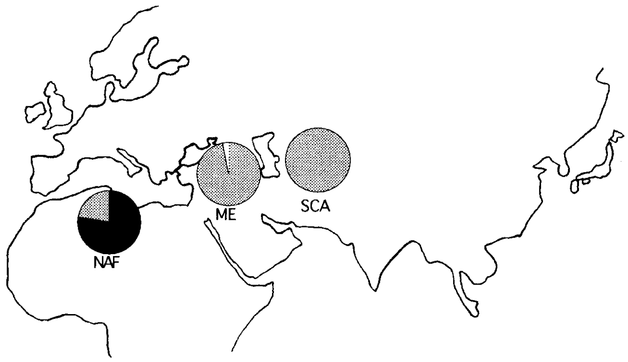
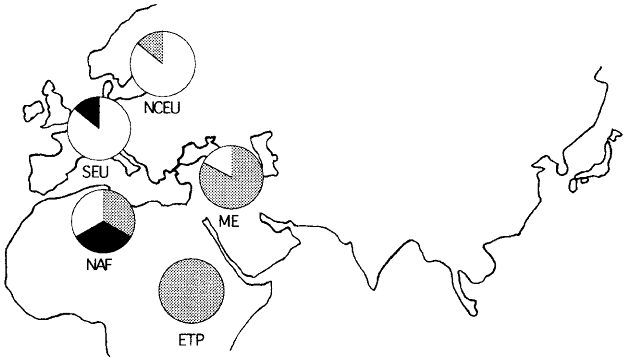


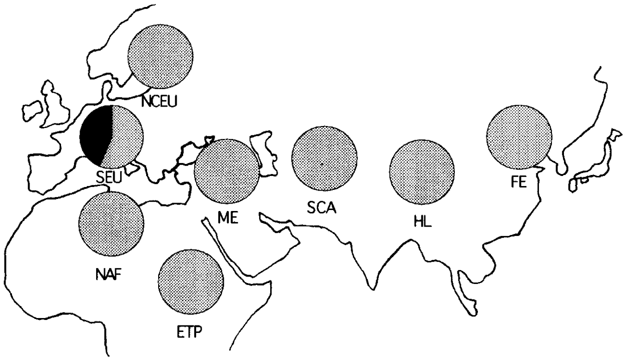
Fig. 1 Restriction site map of cMWG699. The bar indicates the *Taq*I recognition site. The map was made based on Tanno et al. (1999)



a) wild barley (*H. vulgare* ssp. *spontaneum*)



b) two-rowed cultivated barley



c) six-rowed cultivated barley

Fig. 2a–c Geographical distribution of the cMWG699/*Taq*I polymorphism of the three different haplotypes A (*hatched*), K (*open*) and D (*closed*). For abbreviations see Table 1. For information about the genotype in each accession, contact Tanno or Komatsuda

Table 2 Nucleotide polymorphisms at cMWG699 in cultivated and wild barley. Asterisk denotes consensus sequence. Dash denotes gap

Haplotype status		Row	Locality	Accession		Symbol
type A	wild	2 V^p[b]	Turkmenistan	ssp. *spontaneum*	OUH730	I
	wild	2	Turkmenistan	ssp. *spontaneum*	OUH644	
	wild	2	Afganistan	ssp. *spontaneum*	OUH630	
	wild	2	Iran	ssp. *spontaneum*	OUH728	
	wild	2	Iraq	ssp. *spontaneum*	OUH707	
	wild	2V^p[b]	Iraq	ssp. *spontaneum*	OUH742	
	wild	2	Jordan	ssp. *spontaneum*	OUH638	
	wild	2	Turkey	ssp. *spontaneum*	OUH726	
	cult.	2	Iran	Ghazvin 1		
	cult.	2	Chechoslovakia	Jubilee		
	cult.	2	Syria	Aleppo 1		
	cult.	2	Ethiopia	Hiproly		
	cult.	2V^t[b]	Ethiopia	Debre Zeit 29		
	cult.	2V^t[b]	Ethiopia	Glyorgi 1		
	cult.	6	Japan	Azumamugi 3)		I
	cult.	6	China	Paoanchen 1		I
	cult.	6	Tibet	Tibet Violet 1		I
	cult.	6	Afganistan	Kabul 1		I
	cult.	6	Iraq	Baqubah 1		I
	cult.	6	Ethiopia	Adigrat 3		I
	cult.	6	Ethiopia	Addis Ababa 3		I
	cult.	6	Tunisia	Tunis		I
	cult.	6	Italy	Orayio		I
	cult.	6	Finland	Olli[c]		I
type K	wild	2	Iran	ssp. *spontaneum*	OUH729	
	cult.	2	Turkey	Turkey 111		
	cult.	2	Germany	Hanna		
	cult.	2	United Kingdom	Golden Promise[c]		
type D	wild	2	Morocco	ssp. *spontaneum*	OUH777	II
	cult.	2	North African	Palmella Blue		II
	cult.	6	Bulgaria	Caveda		II
	cult.	6	Germany	Dissa[c]		II
	cult.	6	Spain	Valencia		II

[a] The aligned sequences are 934 nucleotides long, including 20-base primer sequences on both ends

[b] V^p and V^t are multiple alleles at *v* (*vrs1*) of two-rowed barleys. Barley of V^p and V^t has tip-awned lateral spikelets (*proskowetzii*) and markedly reduced lateral spikelets (*deficiens*), respectively. All the other two-rowed barleys have *V*

[c] Sequence data of “Azumamugi”, “Olli”, “Golden Promise” and “Dissa” are cited from Tanno et al. (1999)

Sequencing analysis

Sequence analysis was done to reveal further variations of the nucleotide sequence within each haplotype. Since type A was predominant in the restriction analysis (Fig. 2), more accessions of type A were included than those of types K and D. Materials were selected from as distantly separated areas as possible. Table 2 shows polymorphic nucleotide sites of the cMWG699 sequence.

In type A, the ssp. *spontaneum* accessions were more variable than the cultivated accessions, where seven haplotypes were found among eight accessions studied. The two-rowed cultivated barley was less variable, including three haplotypes among six accessions. In contrast, the six-rowed cultivated barleys consisting of ten accessions had an identical sequence. The sequence coincided with that of OUH730, a ssp. *spontaneum* accession from Turkmenistan.

The two-rowed cultivated accessions included two *deficiens* forms from Ethiopia. The *deficiens* barley is two-rowed and has a rudimentary lateral spikelet without any sexual organ (Woodward 1949). The two Ethiopian *deficiens* accessions (“Debre Zeit 29” and “Glyorgi 1”) had the same sequence, which was identical with that of the normal two-rowed cultivars “Aleppo 1” (from Syria) and “Hiproly” (from Ethiopia).

In haplotype K, one wild and three cultivated barleys had an identical sequence (Table 2). Their sequences were also identical with that of “Kanto Nakate Gold” which was analyzed by Tanno et al. (1999). Therefore, all of the type K accessions studied had an identical sequence. The wild accessions originated from Iran (OUH729).

All of the type D barleys analyzed had the same sequence. The six-rowed cultivated barleys analyzed were from Bulgaria (“Caveda”), Germany (“Dissa”) and Spain

Table 2 (continued)

Polymorphic site[a]																	
Exon		Intron													Exon		
64	172	228	242	254	289	297	325	440	448	480	500	509	527	539	732	735	753
C	C	–	C	T	A	A	C	T	G	T	C	T	C	T	T	T	A
*	*	–	*	*	*	*	*	*	*	*	T	*	*	C	C	*	*
*	T	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	–	*	*	*	*	*	*	*	*	*	*	*	*
T	*	–	*	*	*	G	*	*	*	C	*	G	*	*	*	*	*
*	*	–	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	–	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	T	*	*	*	*	C	A	*	*	*	*	*	*	*	*
*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T
*	*	–	*	*	*	*	*	C	A	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	C	A	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	C	A	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	C	A	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*
*	*	–	*	*	*	*	*	C	A	*	*	*	T	*	*	C	*
*	*	–	*	*	*	*	*	C	A	*	*	*	T	*	*	C	*
*	*	–	*	*	*	*	*	C	A	*	*	*	T	*	*	C	*
*	*	–	*	*	*	*	*	C	A	*	*	*	T	*	*	C	*
*	*	–	*	*	*	*	*	C	A	*	*	*	T	*	*	C	*

(“Valencia”). The two-rowed barleys included one cultivated barley from an unknown locality in North Africa (“Palmella Blue”) and one wild barley from Morocco (OUH777).

Discussion

From the results of this study, the six-rowed cultivated barleys were classified into two sequence-based haplotypes, types I and II, at cMWG699 (Table 2). Types I and II correspond to the sequences of the six-rowed barleys that showed types A and D in the restriction analysis (Fig. 2), respectively. The two sequences were the same as in our previous study based on a limited number of cultivated barleys from East Asia, Europe and North America (Tanno et al. 1999). Three scenarios are possible for the differentiation of the six-rowed type I and II barleys: (1) Mutation from two-rowed to six-rowed barleys occurred in the most-recent common ancestor of types I and II barleys, and later cMWG699 evolved to types I and II. (2) Six-rowed barleys of types I and II differentiated from two-rowed barleys of types I and II, respectively, by mutation at *vrs1*. (3) Recombination occurred between *vrs1* and cMWG699, but this probability seems very low under normal cultivation conditions because barley is a highly self-fertile crop and the probability of recombination between the two loci is as low as 1/1,000 (Komatsuda et al. 1999). Our previous study (Tanno et al. 1999) estimated that the DNA sequences of types I and II (types A and D, previously) diverged some 138–830-thousand years ago. This divergence time is much older than the history of barley cultivation (approximately 10-thousand years). This indicates that the six-rowed character of types I and II was most likely derived from independent mutation events from two-rowed barleys of types I and II, respectively.

Helbaek (1959) proposed a theory of the origin of six-rowed cultivated barley based on his study of an archaeological excavation of the Jarmo remains: the two-rowed cultivated barley was first domesticated from wild barley at the foothills somewhere in Kurdistan (Iran, Iraq, Turkey) over 9,000 years ago, and in the 5th millennium, when irrigated agriculture started in the alluvial Mesopotamia and Egypt, six-rowed barley arose from two-rowed cultivated barley by mutation and spread to the artificial ecological environment of the irrigated plain.

In this study, OUH730, a ssp. *spontaneum* from Turkmenistan, had the type I sequence. OUH730, a '*proskowetzii*' form possessing an elongated awn (about 2 cm) at the lateral spikelets, was collected by Dr. F. Bakhteyev (Bakhteyev 1963). The elongated awn is controlled by the V^p allele which is one of the multiple alleles at the *vrs1* locus (Takahashi and Tomihisa 1970). Another '*proskowetzii*' form from Jarmo, Iraq (ssp. *spontaneum* OUH742), has only one base difference from OUH730. These results suggested that the six-rowed character of type I barley had differentiated from the '*proskowetzii*' form, or that the '*proskowetzii*' and the six-rowed forms had differentiated from a common ancestor of type I in this region. Therefore, our data is not incongruent with Helbaek's theory. Six-rowed barley comprised some 90% of cereal crop production in ancient Mesopotamia (Harlan 1970), and probably they were of type I and were spread around the world.

Type II was the other haplotype found in the six-rowed cultivars, which were distributed mainly in the Mediterranean region. This type was also found in two-rowed cultivars from Spain and North Africa (location unknown) and seven Moroccan accessions of ssp. *spontaneum*. These results suggested that the six-rowed barleys of type II probably differentiated from the two-rowed forms of type II in this region. It is, however, still not clear whether the six-rowed barleys were derived from the two-rowed "cultivated" barleys or directly from the ssp. *spontaneum* forms. A number of researchers maintain that the distribution of the Moroccan ssp. *spontaneum* is disjunctive and is different from that of ssp. *spontaneum* in the Middle East where populations of ssp. *spontaneum* form distinctive colonies (Harlan and Zohary 1966; Bothmer et al. 1995). The Moroccan ssp. *spontaneum* accessions collected by Molina-Cano et al. (1982) were more or less weedy plants because their habitat was an artificially disturbed environment occurring along cultivated barley fields. However, a multiple allelic RFLP study by Molina-Cano et al. (1999) demonstrated that the Moroccan ssp. *spontaneum* formed a unique cluster apart from ssp. *spontaneum* of other regions. They pointed out the possibility of the multiple origin of cultivated barley in Morocco. The present study used the Moroccan ssp. *spontaneum* accessions collected by Molina-Cano et al. (1982). If these Moroccan accessions are genuine wild barleys, the two-rowed cultivated barley of type II might have been domesticated from the Moroccan wild barley.

Two-rowed cultivated barley was distributed from the Middle East to Europe, and most of them are of type K. All three cultivated barleys of this type showed the same sequence, and the sequence was identical with that of an Iranian ssp. *spontaneum* accession. The seeds of the Iranian accessions were shrunken and pubescent, characteristics often being observed in ssp. *spontaneum*. However, as the two-rowed spike is common in both cultivated and wild barleys, and introgression between the two forms may occur, it is difficult to assess whether wild barley carried the type K allele from nature or whether the allele was introduced from the two-rowed cultivated barley. For this reason, it is not possible to conclude at this moment that the Iranian wild barley is an original two-rowed cultivated barley of type K.

The *deficiens* form, having V^t allelic at *vrs1*, is two-rowed and has a rudimentary lateral spikelet without any sexual organ (Woodward 1949). Takahashi (1955) speculated that the *deficiens* form was derived recently from normal two-rowed cultivated barley because this form is not found in a wild barley population and the distribution of *deficiens* is limited to a narrow area, such as Ethiopia. In this study, the *deficiens* forms had an identical cMWG699 sequence with that of normal two-rowed cultivated barley from Ethiopia and Syria, and the sequence was not identical with any of ssp. *spontaneum*. This suggested that the *deficiens* form was derived from normal two-rowed barley and supports Takahashi's above-mentioned presumption (Takahashi 1955).

The multiple origin of six-rowed barley suggested in this study is based on the assumption that six-rowed barley differentiated from two-rowed barley, i.e. the two-rowed ancestral theory. The present study demonstrated that variation of the nucleotide sequence was highest in ssp. *spontaneum*, intermediate in the two-rowed cultivated barley and very low in the six-rowed cultivated barley (Table 2). This strongly supports the hypothesis that six-rowed barley was derived from two-rowed barley. The neutrality test based on Tajima's D statistics (Tajima 1989) indicated that the null hypothesis of neutral evolution was discarded for randomly sampled ssp. *spontaneum* (type A accessions) ($D=-1.67$, fitness probability is $p<0.05$). The negative D value implies the existence of a deleterious gene around the DNA region. This is in a good agreement with the observation by Zohary (1963) that six-rowed brittle barleys were apt to be eliminated in a population of the ssp. *spontaneum* form. These pieces of evidence agree with the two-rowed ancestral theory.

In conclusion, this study based on the cMWG699 locus revealed at least two distinct groups in six-rowed cultivated barley, types I and II. The two forms probably differentiated by independent mutations of the *vrs1* locus from two-rowed barleys of types I and II, and the place of their origin may be near Turkmenistan and the Mediterranean region, respectively.

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