Evolutionary process of *Hordeum brachyantherum* 6x and related tetraploid species revealed by nuclear DNA sequences

Takao Komatsuda*1), Björn Salomon2) and Roland von Bothmer2)

1) Plant Genome Research Unit, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
2) Department of Crop Science, The Swedish University of Agricultural Sciences, SE-23053 Alnarp, Sweden

A hexaploid form of *Hordeum brachyantherum* ssp. *brachyantherum* was discovered in California in 1980, and its origin has since been studied over the past three decades. We applied EF-G, a nuclear DNA sequence, to infer the parents of the hexaploid form. In polyploid taxa, amplified DNAs were cloned into a vector, and EF-G copies were amplified from the colonies by PCR and digested with restriction enzymes to separate different types. Phylogenetic analysis was performed based on the DNA sequences. The result showed that *H. brachyantherum* ssp. *brachyantherum* 6x and 4x carried one identical DNA sequence of 910 bp, and had closely related DNA sequences of 931 bp. *H. brachyantherum* ssp. *brachyantherum* 6x and *H. marinum* ssp. *gussoneanum* 2x shared one identical DNA sequence of 915 bp. From these results we hypothesized that *H. brachyantherum* ssp. *brachyantherum* 6x has evolved by an outcrossing between *H. marinum* ssp. *gussoneanum* 2x and *H. brachyantherum* ssp. *brachyantherum* 4x, followed by a chromosome doubling. Our results also indicate that *H. marinum* was involved in the polyploidization of *H. secalinum*, *H. capense*, and *H. marinum*. The origins of *H. jubatum* and *H. depressum* are discussed.

Key Words: Polyploidization, chloroplast translation elongation factor-G (EF-G), speciation, evolution, Triticeae.

Introduction

The genus *Hordeum* L. comprises 32 species (45 taxa), including diploids, tetraploids, and hexaploids, and it has a basic chromosome number of \( x = 7 \) (Bothmer et al. 1995). On the basis of metaphase I chromosome pairing, Bothmer et al. (1986, 1987) suggested the presence of four basic genomes designated \( I \) in *H. vulgare* and *H. bulbosum*, \( X \) (formerly \( Y \)) in *H. marinum*, \( X \) (formerly \( X \)) in *H. marinum*, and \( H \) in the remaining species. We follow this genome designation system in this report. *H. brachyantherum* has two cytotypes, the diploid ssp. *californicum*, which is endemic to California, and the tetraploid ssp. *brachyantherum*, which is distributed over a wide area from western USA and Canada through the Aleutian Islands to Kamchatka (Bothmer et al. 1995). Diploid ancestor of this species probably migrated from South-west Asia, via Beringia, to North America (Blattner 2006). In 1980, one hexaploid (2n = 6x = 42) form was discovered at a small creek in California. It was surrounded by di- and tetraploid populations of the species (Bothmer and Jacobsen 1985). The evolutionary pathways of the hexaploid have not been solved. Its C-banding pattern and meiotic pairing show that it contains an unidentified genome in addition to the genomes of the tetraploid forms (Linde-Laursen et al. 1986b, Bothmer et al. 1989b). Later GISH analysis indicated that the hexaploid carries the genome of *H. marinum* (Taketa et al. 1999). Chloroplast DNA analysis indicated that the hexaploid and *H. marinum* ssp. *gussoneanum* 2x shared an identical DNA sequence (Nishikawa et al. 2002, Jakob and Blattner 2006).

Our objective was to elucidate the parents of the hexaploid of *H. brachyantherum* at the DNA sequence level. We used a nuclear DNA sequence of cMWG699, which harbours a high DNA sequence homology (80%) to the chloroplast translation elongation factor EF-G gene (Hernandez-Torres et al. 1993). cMWG699 is closely linked to the vrs1 locus (two-/six-rowed spike) of barley, *H. vulgare* L. (Graner et al. 1991, Komatsuda et al. 1999a), and the DNA sequence has been used successfully for the phylogenetic study of diploids and tetraploids in *Hordeum* (Komatsuda et al. 1999b, 2001, Tanno et al. 1999, 2002, Petersen and Seberg 2003).

Materials and Methods

Plant material

Most of the plant materials were collected in California (Table 1). *H. depressum* and *H. intercedens* occur in western USA, with the centre of distribution in central California. *H. jubatum* is widely distributed in North America and Siberia. The Californian species are mainly inbreeding, although some outbreeding populations occur in *H. brachyantherum*.

Communicated by N. Mori
Received August 25, 2009. Accepted October 14, 2009.
*Corresponding author (e-mail: takao@affrc.go.jp)
Other than the Californian species, we included *H. marinum* and *H. secalinum* because of their morphological resemblance to *H. brachyantherum* (Bothmer et al. 1995), and *H. capense* because of its similarity to *H. secalinum* (Linde-Laursen et al. 1986a).

*H. marinum* is annual and inbreeding, *H. secalinum* is perennial and more or less outbreeding, and *H. capense* is perennial and mainly inbreeding (Johansen and Bothmer 1994, Cronberg et al. 1997). The wild species are kept at the Department of Crop Science, The Swedish University of Agricultural Sciences, Alnarp, Sweden.

**DNA techniques**

Small-scale isolation of genomic DNA, PCR amplification using the primer pair cMWG699T3-2 and cMWG699T7-2, and restriction digestion of the PCR products followed by separation on 2.2% MetaPhor agarose (FMC, Rockland, Maine, USA) were carried out as described previously (Komatsuda et al. 1998). In polyploid taxa, amplified DNAs were cloned into pCRII vector (Invitrogen, San Diego, California, USA) and EF-G copies were amplified from the colonies by PCR using the same primers and digested with several restriction enzymes to separate different types. Clones from allotetraploid and allohexaploid plants were classified into two and three classes, respectively, on the basis of restriction patterns. We determined the DNA sequences of at least three independent clones for each genome. However, since two genomes did not show a restriction polymorphism in *H. secalinum* and *H. capense*, eight independent clones were sequenced for each species. Such an analysis may result in the error of judging ‘allofetaploid’ as ‘autotetraploid’ at a probability of 0.0078 (=0.5^8-1). The formula explains the probability when all the eight clones show the same sequence in allotetraploids. Dideoxy-termination cycle sequencing was performed using an automated DNA sequencers (PE Applied Biosystems).

**Phylogenetic analysis**

*H. marinum* ssp. *glaucum* (H0076) and *H. vulgare* ssp. *vulgare* (cv. Azumamugi), being representative of the Xu and I genomes, respectively, were included as outgroups on the basis of our previous study (Komatsuda et al. 1999a). Sequence data on these outgroups had been determined previously (Tanno et al. 1999, 2001). DNA sequences were aligned using the Wisconsin Package Version 9.0 (Genetic Computer Group (GCG), Madison, Wisconsin, USA) by setting a gap weight of 5 and gap length weight of 1. Cladistic analysis (maximum parsimony) and bootstrapping of 1000 replicates were performed with PAUP* 4.0b10 (Swofford 2002). A heuristic search procedure was performed with tree-bisection-reconnection (TBR) branch-swapping algorithm. Gaps were treated as missing and character-state optimization was the accelerated transformation (ACCTRAN).
Results

Restriction patterns of amplified DNAs

Figure 1A shows DNAs amplified from genomic DNAs of diploid, tetraploid, and hexaploid plants. Figure 1B shows the TaqI-restriction patterns of the amplified DNAs. *H. marinum* ssp. *gussoneanum* 4x, *H. brachyantherum* ssp. *brachyantherum* 4x and 6x, and *H. jubatum* showed heterogeneous bands. The patterns demonstrated that *H. marinum* ssp. *gussoneanum* 4x had two classes of DNA amplicon: one showed a restriction pattern identical to that of ssp. *gussoneanum* 2x and the other showed a unique pattern. The two patterns were identical to the ones of the Eurasian lines of *H. marinum* ssp. *gussoneanum* 4x (Komatsuda et al. 2001). *H. brachyantherum* ssp. *brachyantherum* 4x and 6x had two and three classes of DNA amplicon, respectively, as shown by DNA sequences (Table 1). Two accessions (H2001 and H2421) of the hexaploid form showed an identical pattern, probably because they were collected at the same site in California but at different times, or as 6x *brachyantherum* is a young taxon with not much variation. Hence, we included only H2421 in the sequence analysis.

The restriction patterns of *H. intercedens* and *H. depressum* were identical. *H. jubatum*, being tetraploid, had two classes of DNA amplicon. *H. secalinum* showed a restriction pattern identical to that of *H. marinum* ssp. *gussoneanum* 2x. The restriction pattern of *H. capense* was unique, but it resembled that of *H. marinum* ssp. *gussoneanum* 2x.

Homologous DNAs in polyploid forms

Two classes of clone were detected in *H. marinum* ssp. *gussoneanum* 4x, *H. brachyantherum* ssp. *brachyantherum* 4x, and *H. jubatum*, and three classes of clone in *H. brachyantherum* ssp. *brachyantherum* 6x (Table 1). These results were congruent with the restriction patterns (Fig. 1B).

*H. depressum*, *H. secalinum*, and *H. capense*, tetraploid species, showed one class of clone by the restriction analysis, so eight independent clones were sequenced to find sequence polymorphism between two genomes in each species. Two classes were identified in *H. depressum*: three clones of 931 bp and five clones of 930 bp. However, only one class of clones was detected in *H. secalinum* and *H. capense* (Table 1).

Phylogenetic analysis

A heuristic search was performed to find the most parsimonious trees. Of 962 total characters, 867 were constant, 41 variable characters were parsimony-uninformative, and 54 characters were parsimony-informative (ESM1). All characters were equally weighted. A total of 154 equally most parsimonious trees (length = 110) were retained. A strict consensus tree, consisted of two large groups, separating sequences derived from North American and Eurasian taxa (Fig. 2).

The North American group contained *H. brachyantherum*, *H. depressum*, *H. jubatum*, and *H. intercedens*, and the clade was supported by a bootstrap value of 85%. *H. brachyantherum* ssp. *californicum* was separated from all the other members of this group. There were three clades, where different genomes of polyploid species were mutually superimposed. The first clade included *H. brachyantherum* ssp. *brachyantherum* 4x and 6x (931 bp for each), *H. depressum* (930 bp) and *H. jubatum* (957 bp). The second
clade was highly supported by a bootstrap value of 100%. *H. brachyantherum* ssp. *brachyantherum* 4x and 6x shared an identical DNA sequence of 910 bp. *H. jubatum* had only a single nucleotide change from *H. brachyantherum* ssp. *brachyantherum* 4x and 6x, indicating that these taxa carry very similar genomes. The third clade was also highly supported, including *H. depressum* and *H. intercedens*.

The Eurasian group contained *H. marinum* ssp. *marinum* and ssp. *gussoneanum*, *H. capense*, *H. secalinum*, and one DNA fragment of *H. brachyantherum* ssp. *brachyantherum* 6x (915 bp). The 915-bp fragments were common in *H. marinum* ssp. *gussoneanum* 2x and 4x, *H. brachyantherum* ssp. *brachyantherum* 6x, and *H. secalinum*, showing an identical DNA sequence (Fig. 2). *H. capense*, *H. marinum* ssp. *marinum*, and the 931-bp fragment of *H. marinum* ssp. *gussoneanum* 4x formed a highly supported clade.

**Discussion**

This study revealed that *H. brachyantherum* ssp. *brachyantherum* 6x carries two genomes, which are homologous to those of *H. brachyantherum* ssp. *brachyantherum* 4x. The two genomes of *H. brachyantherum* ssp. *brachyantherum* 4x and 6x are regarded as diverse (Linde-Laursen 1986b, Bothmer et al. 1989b, Taketa et al. 1999). Our results suggest that the genome harbouring the 931-bp DNA is diverse between the two taxa (Fig. 2). It is unclear whether the difference in the DNA sequence occurred before or after the evolution of the hexaploid cytotype.

*H. jubatum* and *H. brachyantherum* are genetically very similar (Bothmer et al. 1987, 1988). The two species have an overlapping distribution area in which hybrid swarms are common (Bothmer et al. 1995). Our study showed that *H. jubatum* and *H. brachyantherum* 4x share two genomes (Fig. 2); one of the genomes is divergent to some extent, including a 26-bp tandem duplication of CTTTTAT TCTTTTTCTTTTTCGGTCA in the 957-bp fragment in *H. jubatum*. *H. depressum* is considered to be allotetraploid, evolved by a cross between *H. brachyantherum* ssp. *californicum* and *H. pusillum* (Covas 1949) or *H. intercedens* (Baum and Bailey 1988, 1991, Salomon and Bothmer 1998). Our result is congruent with the postulation that *H. intercedens* was a parent of *H. depressum*. Recently, chloroplast DNA sequence analysis has suggested that *H. depressum* and *H. brachyantherum* ssp. *californicum* have a common ancestor (Nishikawa et al. 2002, Jakob and Blattner 2006); therefore, *H. brachyantherum* ssp. *californicum* was probably a maternal parent and...
H. intercedens was probably a paternal parent of H. depressum (Salomon and Bothmer 1998).

It has been shown by GISH analysis that H. brachyantherum ssp. brachyantherum 6x carries 14 chromosomes derived from H. marinum, but whether they are derived from ssp. marinum or ssp. gussoneanum was not specified (Taketa et al. 1999). Our results revealed the origin to be H. marinum ssp. gussoneanum 2x. H. marinum is mainly distributed in western and central Europe, the Mediterranean, and Middle and South-western Asia (Bothmer et al. 1989a). Having been introduced to the New World, H. marinum ssp. gussoneanum 2x and 4x occur in California, where H. brachyantherum 6x was discovered (Bothmer and Jacobsen 1985). H. brachyantherum ssp. brachyantherum 4x is mainly inbreeding, but some more or less outbreeding populations occur. These ecogeographical evidences, together with the present results, support the hypothesis that H. brachyantherum ssp. brachyantherum 6x evolved from a hybridization between H. marinum ssp. gussoneanum 2x and H. brachyantherum ssp. brachyantherum 4x, followed by a chromosome doubling. Recently, chloroplast DNA sequence analysis clearly demonstrated that two lines of H. brachyantherum ssp. brachyantherum 6x and seven lines of H. marinum ssp. gussoneanum 2x shared an identical DNA sequence (Nishikawa et al. 2002, Jakob and Blattnier 2006); therefore, H. marinum ssp. gussoneanum 2x seems to be a maternal parent of the hexaploid.

H. secalinum has been considered as allotetraploid (Bothmer et al. 1995). H. secalinum carries the genome of H. marinum and the genome of an unidentified diploid species belonging to the H genome group (Taketa et al. 1999). Our results revealed that H. secalinum carries the genome of H. marinum ssp. gussoneanum 2x, which agrees in part with the observation by Petersen and Seberg (2004). We could not identify the other genome of H. secalinum, probably because the cloning procedure allowed it to escape detection. Blattner (2004) also reports that he couldn’t find ITS copies derived from H. marinum/gussoneanum in H. capense, and only one clone in his H. secalinum sample. With EF-G the pattern seems reversed but might somehow indicate that some rearrangements within the genomes took place after intergenomic polyploidization as suggested by Taketa et al. (2009). Petersen and Seberg (2004) distinguished two genomes, where one was H. marinum ssp. gussoneanum 2x and the other genome was similar to diploid species of H genome. It has already been shown that H. marinum ssp. gussoneanum 4x carries the genome of H. marinum ssp. gussoneanum 2x and the genome of an unidentified diploid species (Komatsuda et al. 2001). Our results showed that H. brachyantherum ssp. brachyantherum 6x, H. marinum ssp. gussoneanum 4x, and H. secalinum also carry the genome of H. marinum ssp. gussoneanum 2x, probably in an intact form. The origin of the second DNA fragment (931 bp) of H. marinum ssp. gussoneanum 4x was unknown (Komatsuda et al. 2001); our results show that it formed a clade with H. marinum ssp. marinum and H. capense.

The chloroplast DNA study by Nishikawa et al. (2002) and Jakob and Blattner (2006) indicated a close relationship between H. marinum ssp. marinum and H. secalinum, whereas our results suggest a closer relationship between H. marinum ssp. gussoneanum and H. secalinum, because they share identical sequences (although without any synapomorphy). As pointed out by Petersen and Seberg (2003), the discrepancy between plastid and nuclear DNA data is most probably due to lineage sorting. Nevertheless, it is clear that H. marinum ssp. gussoneanum has a unique position of involvement in the formation of several polyploid species and taxa in Hordeum, acting as maternal parent. Chloroplast variation was completely absent within the cytotypes of H. marinum ssp. gussoneanum, indicating a severe and recent genetic bottleneck (Jakob et al. 2007). Our study supports the hypothesis that H. marinum, most particularly ssp. gussoneanum, has a genetic mechanism for polyploidization (Bothmer et al. 1989a). Because chloroplast DNA study has revealed a considerable divergence of cytoplasmic genomes between H. marinum ssp. marinum and ssp. gussoneanum (Nishikawa et al. 2002), the genetic mechanism for polyploidization seems to be inherited by the nuclear genome of H. marinum ssp. gussoneanum.

Acknowledgements

This study was supported by grants for the “Bilateral International Joint Research Coordination” (13073-2125-14) from the Ministry of Education, Culture, Sports, Science and Technology, Japan; for the “Core Research for Evaluational Science and Technology (CREST)” from the Japan Science and Technology Corporation (JST), Japan; and for the “Genomics for Agricultural Innovation (TRC1004)” from the Ministry of Agriculture, Forestry and Fisheries of Japan.

Literature Cited


