Starch is a biologically and commercially important polymer of glucose.\textsuperscript{1–3} It is synthesized to form starch grains (SGs) inside amyloplasts of higher plants. Because starch is clearly stained by iodine solution, the subcellular morphologies of SGs can be easily observed under the microscope.\textsuperscript{4–6} Despite the simple composition of glucose polymers, SGs exhibit various morphologies depending on plant species.\textsuperscript{5–12} Morphologically, SGs are classified as either compound or simple grains. Compound grains consist of many starch granules in a single amyloplast, and simple grains are composed of a single starch granule in an amyloplast. The endosperm of Poaceae species accumulates high levels of starch up to more than 90% of total weight. Previously published work classified Poaceae species into four types based on the SG morphologies of endosperms.\textsuperscript{8–12} Species that have a compound grain type such as rice (\textit{Oryza sativa}) possess only compound grains in their endosperms. Species that have a simple grain type are further divided into two subtypes referred to as bimodal and uniform. The bimodal type produces both small and much larger simple SGs that coexist in the same cells. The uniform type possesses similarly sized hexagonal, pentagonal, round and simple SGs. For example, barley (\textit{Hordeum vulgare}) and wheat (\textit{Triticum aestivum}) develop bimodal SGs, and uniform SGs are found in maize (\textit{Zea mays}). In the fourth type of SG morphology, a number of species of \textit{Miscanthus}, \textit{Peroxis}, \textit{Gymnopogon} and \textit{Thuarea} display a mixed configuration of compound and simple grains inside the same cell of the endosperm.\textsuperscript{8,9} Despite the comprehensive observations of SG morphologies in \textit{ planta}, a detailed examination of the phylogenetic origin of the morphological diversities of SGs is lacking. It is not known which SG morphological type is plesiomorphic in the family Poaceae, or how SG morphological types are clustered and scattered in the phylogenetic tree. In this study, we evaluated the previous observations of SG morphologies in the Poaceae and arranged these data on the most current phylogenetic tree of the Poaceae. Furthermore, we prepared thin sections of endosperms and observed SG morphologies of 26 species belonging to the four genera \textit{Hordeum}, \textit{Elymus}, \textit{Triticum} and \textit{Bromus}, which were found to be clustering in the phylogenetic tree. In previous work, these four genera were considered to exclusively contain the bimodal simple grain type. However, this study showed that \textit{Bromus} species contained remarkable intrageneric variation. This result contrasted with data for the other three genera, which contained only bimodal simple grains. The phylogenetic analysis performed in this study will contribute to a greater understanding of SG morphological diversity.

Key words: \textit{Bromus}, endosperm, phylogenetic analysis, Poaceae, starch grain
understanding of SG morphological diversity.

MATERIALS AND METHODS

Plant materials. All plant materials used for observing SG morphology and analysis of DNA are listed in Table 1. Each seed material used for SG analysis was of the same accession that was used for DNA extraction. Barley accessions of *Hordeum vulgare* ssp. *vulgare* and *H. vulgare* ssp. *spontaneum* were provided by the National BioResource Project for barley of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The seeds of *Triticum boeoticum*, *T. urartu*, *T. monococcum*, *T. spelta* and *T. macha* were provided from Mr. Takakazu Matsuura (Institute of Plant Science and Resources, Okayama University, Japan). Other seeds were preserved in the herbaria of the Institute of Plant Science and Resources and the Kura-shiki Museum of Natural History (KURA) in Japan.

Preparation of endosperm thin sections and microscopy. Approximately 1 mm cubic blocks were cut out from the endosperm of dry seeds and fixed in FAA solution containing 5% (v/v) formalin, 5% (v/v) acetic acid and 50% (v/v) ethanol for at least 12 h at room temperature. Samples were subsequently dehydrated through a graded ethanol series [30% (v/v), 50, 70, 90 and 100%], and then embedded in Technovit 7100 resin (Kulzer and Company Inc., Wehrheim, Germany). The embedded samples were cut in 1 µm sections with an ultramicrotome (LEICA EM UC7, Leica Microsystems K.K., Tokyo, Japan) and diamond knives, and then dried on coverslips. Thin sections were stained with 40-times diluted Lugol solution in deionized water for at least 5 s and subsequently examined under a microscope (AX70, Olympus Co., Tokyo, Japan).

DNA extraction and phylogenetic analysis. DNA sequences were analyzed for the same seed collections as those used for observation of SGs. Total genomic DNA was extracted from frozen seeds using the small-scaled CTAB method with modifications. One to three seeds were used for each plant material. Two neighboring regions on plastid DNA, the *trnL* intron and the *trnL-trnF* intergenic spacer (IGS), were amplified using PCR. To amplify the DNA region, we used a pair of PCR primers designed by Taberlet et al. (1991) (5'-(CGAAATCGGTAGACGCTACG-3' and 5'-ATTTGAACTGACGAAGGATaac-3')) and the following PCR temperature profile: initial denaturation at 94°C for 2.5 min; a 37 cycle reaction with denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min; and final extension at 72°C for 5 min. The PCR products were purified with MicroSpin S-300HR Columns (GE Healthcare UK Ltd., Hertfordshire, UK) to remove the primers and dNTPs. The purified double-stranded PCR products were used as templates for direct sequencing by the dyeoxy method using a genetic analyzer (Applied Biosystems 3130xl) and Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Ltd., Foster, USA) according to the manufacturer’s instructions. For sequencing complementary strands of the plastid DNA regions, the following two primers designed by Taberlet et al. (1991) (5'-(GGTCCAAGTCCCTCTATCCC-3' and 5'-GGGGATAGAGGGACTTGAAC-3')) were used in addition to the former two primers. All sequences were aligned manually with a text editor. A phylogenetic tree was constructed using the neighbor-joining (NJ) method and the computer software MEGA v. 4.0.2. The evolutionary distances were computed using the maximum composite likelihood method and are reported in units of the number of base substitutions per site. All nucleotide sites containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

RESULTS AND DISCUSSION

Phylogenetic evaluation of starch grain morphologies in the family Poaceae.

Previous observations of SG morphologies were arranged onto the latest phylogenetic tree of the family Poaceae (Fig. 1). We used the Bayesian consensus phylogenetic tree constructed by the Grass Phylogeny Working Group II, which is based on DNA sequences of three marker genes. The phylogenetic tree covers 545 Poaceae species of 314 genera. The previous data of SG morphologies that were used for plotting the molecular phylogeny were derived from the two comprehensive studies of Tateoka (1962) and the Grass Phylogeny Working Group (2001). Tateoka’s study includes 766 species belonging to 244 genera; the Grass Phylogeny Working Group’s study includes 76 species from 76 genera. Because most previous observations only describe the genus names but not the species, we combined branches of species belonging to the same genera in the phylogenetic tree (Fig. 1, JSTAGE Supplementary Material, Figs. S1 (A), (B)). According to the previous observations, the names of genera are colored depending on the morphological types of SGs: compound grains are red, bimodal simple grains are blue, uniform simple grains are green and a mixed configuration of compound and simple grains is yellow. In the genera highlighted with gray, discrepancies are found between observations or several types of SGs were reported in the same observation. We cannot exclude the possibility that the different observations caused the discrepancies, however, it is also possible that intrageneric variations of the SG morphological types are exist in the gray genera.

Figure 1 is a most suggestive part of the phylogenetic tree. The molecular phylogeny indicates that early-diverging genera in the Poaceae such as *Pharus*, *Anomochloa* and *Streptochaeta* develop compound SGs (Fig. 1). This indicates that the compound grain type is the ancestral state of SG morphologies in the Poaceae. This result is consistent with the previous study performed by the Grass Phylogeny Working Group.

Some genetic modifications that occurred during speciation probably generated the other SG morphological types from the compound grain type.

The details of PACMAD (a clade includes grass subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae) are described in JSTAGE Supplementary Material, Fig. S1. Genera with the uniform simple grain type were scattered across phylogenetic branches, and this type was most frequent within the tribes Paniceae, Andropogoneae and Paspaleae (JSTAGE Supplementary Material, Fig. S1 (B)). In contrast, the bimodal simple grain type was restricted to only five genera, including *Brachypodium*, *Triticum*, *Hordeum*, *Elymus* and *Bromus* (Fig. 1). Of these, *Triticum*, *Hordeum*, *Elymus* and *Bromus* are clustered as a clade composed of the tribes Bromaeae and
Table 1. Materials for starch grain and DNA sequence analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>Collectors</th>
<th>Seed acc. no.*</th>
<th>Voucher no. *</th>
<th>DDBJ acc. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bromus carinatus</em> Hook. et Arn.</td>
<td>JAPAN: Kojima-shio-nasu, Kurashiki, Okayama Pref., 3 m alt.</td>
<td>S. Kariyama</td>
<td>KURA-8841</td>
<td></td>
<td>AB732921</td>
</tr>
<tr>
<td><em>B. catharticus</em> Vahl</td>
<td>JAPAN: Tamashima-otoshima, Kurashiki, Okayama Pref., 2 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-9448S</td>
<td>RIB-30773</td>
<td>AB732922</td>
</tr>
<tr>
<td><em>B. commutatus</em> Schrad.</td>
<td>JAPAN: Koyou-cho-higashi, Kobe, Hyogo Pref., 3 m alt.</td>
<td>M. Mizuta</td>
<td>KURA-47423</td>
<td></td>
<td>AB732923</td>
</tr>
<tr>
<td><em>B. diandrus</em> Roth</td>
<td>JAPAN: Okayama Harbor, Okayama Pref., 2 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-15487S</td>
<td>RIB-59661</td>
<td>AB732924</td>
</tr>
<tr>
<td><em>B. hordeaceus</em> L.</td>
<td>JAPAN: Okayama Harbor, Okayama Pref., 2 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-15488S</td>
<td>RIB-59663</td>
<td>AB732925</td>
</tr>
<tr>
<td><em>B. remotiflorus</em> (Steud.) Ohwi</td>
<td>JAPAN: Oosa, Niimi, Okayama Pref.</td>
<td>H. Yamane</td>
<td>RIB-15278S</td>
<td>RIB-58529</td>
<td>AB732926</td>
</tr>
<tr>
<td><em>B. secalinus</em> L.</td>
<td>JAPAN: Okayama Harbor, Okayama Pref., 2 m alt.</td>
<td>H. Kobatake</td>
<td>KURA-8415</td>
<td></td>
<td>AB732927</td>
</tr>
<tr>
<td><em>B. tectorum</em> L.</td>
<td>JAPAN: Okayama Harbor, Okayama Pref.</td>
<td>H. Kobatake</td>
<td>RIB-14420S</td>
<td>RIB-30195</td>
<td>AB732928</td>
</tr>
<tr>
<td><em>Elymus caninus</em> L.</td>
<td>JAPAN: Mt. Ibuki, Shiga Pref., 1270 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-11118S</td>
<td>RIB-41608</td>
<td>AB732929</td>
</tr>
<tr>
<td><em>E. dahuricus</em> Turcz. ex Griseb.</td>
<td>JAPAN: Monomyama-cho-tango, Kyoto, Kyoto Pref., 10 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-13056S</td>
<td>RIB-48374</td>
<td>AB7329</td>
</tr>
<tr>
<td><em>E. humidus</em> Osada</td>
<td>JAPAN: Monomyama-cho-tango, Kyoto, Kyoto Pref., 10 m alt.</td>
<td>T. Fujii</td>
<td>RIB-11352S</td>
<td>RIB-46735</td>
<td>AB732931</td>
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<tr>
<td><em>E. racemifer</em> Tsvel.</td>
<td>JAPAN: Mt. Tanematsu, Kurashiki, Okayama Pref.</td>
<td>T. Enomoto</td>
<td>RIB-14660S</td>
<td>RIB-58164</td>
<td>AB732932</td>
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<tr>
<td><em>E. tsukusiensis</em> Honda var. <em>transiens</em> Osada</td>
<td>JAPAN: Tamashima-otoshima, Kurashiki, Okayama Pref., 1 m alt.</td>
<td>T. Enomoto</td>
<td>RIB-9453S</td>
<td>RIB-30768</td>
<td>AB732933</td>
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<tr>
<td><em>Hordeum bulbosum</em> L.</td>
<td>Unknown</td>
<td>K. Tanno</td>
<td>RIB-15343S</td>
<td>no specimen</td>
<td>AB732934</td>
</tr>
<tr>
<td><em>H. hyrix</em> Roth</td>
<td>JAPAN: Tamashima-otoshima, Kurashiki, Okayama Pref.</td>
<td>H. Kobatake</td>
<td>RIB-8480S</td>
<td>no specimen</td>
<td>AB732935</td>
</tr>
<tr>
<td><em>H. marinum</em> L.</td>
<td>JAPAN: Shiba-machi, Yokohama, Kanagawa Pref.</td>
<td>N. Kaneko</td>
<td>RIB-13126S</td>
<td>RIB-52346</td>
<td>AB732936</td>
</tr>
<tr>
<td><em>H. pusillum</em> Nutt.</td>
<td>JAPAN: Okayama Harbor, Okayama Pref.</td>
<td>H. Kobatake</td>
<td>RIB-1720S</td>
<td>no specimen</td>
<td>AB732937</td>
</tr>
<tr>
<td><em>H. vulgare</em> L. ssp. spontaneum K. Koch</td>
<td>Unknown</td>
<td>Unknown</td>
<td>H602</td>
<td>no specimen</td>
<td>AB732938</td>
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<tr>
<td><em>H. vulgare</em> ssp. vulgar cv. Harunamijo</td>
<td>Unknown</td>
<td>Unknown</td>
<td>J247</td>
<td>no specimen</td>
<td>AB732939</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>Unknown</td>
<td>K. Tanno</td>
<td>RIB-15296S</td>
<td>no specimen</td>
<td>AB732940</td>
</tr>
<tr>
<td><em>T. boeoticum</em> Boiss.</td>
<td>Unknown</td>
<td>Unknown</td>
<td>RIB-1-1</td>
<td>no specimen</td>
<td>AB732941</td>
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<tr>
<td><em>T. durum</em> Desf.</td>
<td>Unknown</td>
<td>K. Tanno</td>
<td>RIB-15366S</td>
<td>no specimen</td>
<td>AB732942</td>
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<tr>
<td><em>T. macha</em> Dekaprel. et Menabde</td>
<td>Unknown</td>
<td>Unknown</td>
<td>RIB-1-8-1</td>
<td>no specimen</td>
<td>AB732943</td>
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<tr>
<td><em>T. monococcum</em> L.</td>
<td>Unknown</td>
<td>Unknown</td>
<td>RIB-3-1</td>
<td>no specimen</td>
<td>AB732944</td>
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<tr>
<td><em>T. spelta</em> L.</td>
<td>Unknown</td>
<td>Unknown</td>
<td>RIB-1-9-1</td>
<td>no specimen</td>
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<tr>
<td><em>T. urartu</em> Thumanjan ex Gandilyan</td>
<td>Unknown</td>
<td>Unknown</td>
<td>RIB-2-1</td>
<td>no specimen</td>
<td>AB732946</td>
</tr>
</tbody>
</table>

*Except for three species (*Bromus carinatus*, *B. commutatus* and *B. secalinus*), seed collections with accession numbers were used for DNA analysis and microscopic observation. The voucher number is the accession number of voucher specimen that is a herbarium specimen collected from the same population of the seed collection, if it is preserved. The voucher numbers of the specimens at Institute of Plant Science and Resources, Okayama University (IPSBR) have "RIB-" prefixedly. "RIB" is the abbreviation of "Research Institute for Bioresources", previous name of IPSR. The analyzed seeds of *B. carinatus*, *B. commutatus* and *B. secalinus* were picked from the voucher specimen preserved at the Kurashiki Museum of Natural History (KURA).
Triticeae (Bromeae + Triticeae). The Bromeae + Triticeae clade is sister to the Poeae1 + Poeae2 (Poeae) clade that develops compound grains. Bromeae, Triticeae and Poeae form a single clade that is a sister clade to the tribe Brachypodieae. The genus *Melica* (tribe Meliceae), which is the sister group of the clade composed of the four tribes Bromeae, Triticeae, Poeae and Brachypodieae, is undergoing development of a compound grain (Fig. 1). This phylogenetic relationship suggests two evolutionary scenarios with respect to SG types. The first scenario is that genetic modifications that changed compound grains to bimodal simple grains occurred independently twice: once during the development of Brachypodieae, and once during the development of a common ancestor of Bromeae and Triticeae. The second scenario is that the first genetic modification occurred during the development of a common ancestor of Bromeae, Triticeae, Poeae, and Brachypodieae that changed the compound grain to the bimodal simple grain; thereafter, a second genetic modification occurred during the development of Poeae that reversed the bimodal simple grain to the compound grain. Both scenarios assume that two genetic modifications occurred. There is no current information to enable the assessment of which evolutionary scenario is more probable, the development of compound grains to bimodal simple grains or the reverse. The genetic mechanisms that change SG grain morphological types should be clarified.

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**Fig. 1.** A phylogenetic tree of the family Poaceae classified with starch grain (SG) morphological types. This phylogenetic tree was modified from the Bayesian consensus phylogenetic tree constructed by Grass Phylogeny Working Group II (2012). The names of genera are colored depending on the morphological types of SGs: compound grains are red, bimodal simple grains are blue, uniform simple grains are green and a mixed configuration of compound and simple grains is yellow. Genera that have discrepancies between observations and genera in which several types of SGs were reported are highlighted with gray. On the details of PACMAD clade, see JSTAGE Supplementary Material, Fig. S1. BEP, the subfamilies Bambusoideae, Ehrhartoideae and Pooideae; PACMAD, the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae.
in future studies to determine the correct SG morphology evolutionary scenario.

**Interspecific morphological variations of starch grains in Bromus species.**

The phylogenetic re-evaluation showed that Triticum, Hordeum, Elymus and Bromus are bimodal simple grain types and they are clustered in the phylogenetic tree (Fig. 1). Because the bimodal simple grain type is limited to five genera even in the previously published observations, the genera should be characteristic in the SG phylogenetic morphology. The previously published observations did not provide the species names of each genus and did not show clear images of SGs. Therefore, we prepared clear images of SGs of these genera by collecting seeds of each genus and preparing thin sections of endosperms.

We collected seeds of six Hordeum species including one subspecies (H. murinum, H. hystrix, H. bulbosum, H. vulgare, H. vulgare ssp. vulgare and H. vulgare ssp. spontaneum), five Elymus species (E. caninus, E. racemifer, E. humidus, E. dahuricus and E. tsukusiensis), seven Triticum species (T. monococcum, T. boeoticum, T. urartu, T. durum, T. spelta, T. macha and T. aestivum), and eight Bromus species (B. catharticus, B. carinatus, B. remotiflorus, B. tectorum, B. diandrus, B. hordeaceus, B. commutatus and B. secalinus). In this study, the same seed collections were used for DNA sequencing and SG analyses for construction of the phylogenetic tree. The aligned matrix consisted of 1,168 nucleotide sites. Among these, 225 sites included gaps, 1,101 sites were constant, 67 sites were variable, and 48 sites were phylogenetically informative. All analyzed genera including Bromus, Elymus, Hordeum and Triticum formed a monophyletic group within the NJ tree, respectively. This result is based on the DNA sequences and phylogenetic analysis. The optimal tree with the sum of branch length = 0.06785799 is shown in Fig. 2.

Iodine staining enabled clear visualization of SG morphologies in the endosperm thin sections (Figs. 3–6). All analyzed species belonging to the Hordeum, Elymus and Triticum genera showed the bimodal simple grain type, consistent with previous observations (Figs. 3–5). The percentage of smaller and larger SGs varied depending on species. In H. pusillum, small SGs are highly abundant compared with other species (Fig. 3 (D)). In contrast, small SGs are rare in E. caninus (Fig. 4 (A)). Genetic factors controlling the numbers of small and large SGs have not been identified so far. However, small SGs are known to lack in a few wild wheat species (Aegilops) and a major QTL controlling the small SGs number has been detected. The same genetic mechanism may underlie the interspecific variations between the bimodal simple grain species.

Morphological variations of SGs were found at the intragenic level in Bromus species (Fig. 6). Out of eight analyzed species, only B. catharticus contained the bimodal simple SGs (Fig. 6 (A)). The other species contained uniform-sized simple SGs (Figs. 6 (B)–(H)). This result contrasts with previously published data. B. catharticus and B. carinatus were very closely related to each other in the phylogenetic tree (Fig. 2); however, their SG morphologies were dramatically different (Figs. 6 (A) and (B)). The sizes of observed SGs also varied depending on species. B. diandrus contained the largest SGs, whereas B. hordeaceus contained...
Fig. 3. Iodine-stained thin sections of endosperms from *Hordeum* species.


Fig. 4. Iodine-stained thin sections of endosperms from *Elymus* species.

(A) *E. caninus*, (B) *E. racemifer*, (C) *E. humidus*, (D) *E. dahuricus* and (E) *E. tsukusiensis*. Bar = 20 µm.

Fig. 5. Iodine-stained thin sections of endosperms from *Triticum* species.

(A) *T. monococcum*, (B) *T. boeoticum*, (C) *T. urartu*, (D) *T. durum*, (E) *T. spelta*, (F) *T. macha* and (G) *T. aestivum*. Bar = 20 µm.

Fig. 6. Iodine-stained thin sections of endosperms from *Bromus* species.

(A) *B. catharticus*, (B) *B. carinatus*, (C) *B. remontiflorus*, (D) *B. tectorum*, (E) *B. diandrus*, (F) *B. hordeaceus*, (G) *B. commutatus* and (H) *B. secalinus*. Bar = 20 µm.
the smallest SGs among the eight Bromus species (Figs. 6 (E) and (F)). Size variations of SGs did not correspond to the phylogenetic relationships, as observed for B. tectorum and B. diandrus (Figs. 2, 6 (D) and 6 (E)). This means that small number of genes determine the interspecific size variation of SGs. These are the first clear images showing interspecific SG morphological variation in a single genus.

Many starch-related mutants have been isolated in many plant species by both forward and reverse genetic approaches. The analysis of such mutants has clarified the universal starch biosynthetic processes. Some such starch-related mutants exhibit different SG morphologies than wild-type strains; however, a molecular explanation for the interspecific morphological differences of SGs has not been proposed. A simple SG is formed as a single SG in an amyloplast in which the SG occupies most of the volume of the amyloplast. Therefore, the amyloplast size will be closely correlated with the simple SG size. The amyloplast is a specialized form of a differentiated plastid, as is the chloroplast. The size of a plastid or chloroplast is regulated by mechanisms regulating organelar division, especially ring structures at the division sites. Many mutants that are defective in chloroplast division develop enlarged chloroplasts in their leaves. However, mechanisms regulating amyloplast size are unknown because amyloplast division is suggested to be different from that of chloroplast. Recently, we isolated several rice novel mutants defective in SG morphologies using a rapid observation method for SGs. One of the mutants, ssg4, contained enlarged amyloplasts. A molecular genetic analysis of ssg4 to identify the factors responsible for amyloplast size control is currently in progress. A diversity of such molecular factors may explain the size variation of SGs in Bromus species.

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The authors would like to thank the National BioResource Project for barley of the MEXT, Japan. We especially thank Dr. Kazuhiro Sato (Institute of Plant Science and Resources, Okayama University) for providing the seeds of two subspecies of H. vulgare. We also would like to thank Mr. Takakazu Matsuura (Institute of Plant Science and Resources, Okayama University) for providing the seeds of Triticum species. Finally, we want to sincerely thank Dr. Elizabeth A. Kellogg (University of Missouri-St. Louis) for giving us the important information of the phylogenetic tree of the family Poaceae.

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