

High frequency of HMW-GS sequence variation through somatic hybridization between *Agropyron elongatum* and common wheat

Xin Gao · Shu Wei Liu · Qun Sun · Guang Min Xia

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Abstract A symmetric somatic hybridization was performed to combine the protoplasts of tall wheatgrass (*Agropyron elongatum*) and bread wheat (*Triticum aestivum*). Fertile regenerants were obtained which were morphologically similar to tall wheatgrass, but which contained some introgression segments from wheat. An SDS-PAGE analysis showed that a number of non-parental high-molecular weight glutenin subunits (HMW-GS) were present in the symmetric somatic hybridization derivatives. These sequences were amplified, cloned and sequenced, to deliver 14 distinct HMW-GS coding sequences, eight of which were of the y-type (*Hy1–Hy8*) and six x-type (*Hx1–Hx6*). Five of the cloned HMW-GS sequences were successfully expressed in *E. coli*. The analysis of their deduced peptide sequences showed that they all possessed the typical HMW-GS primary structure. Sequence alignments indicated that *Hx5* and *Hy1* were probably derived from the tall wheatgrass genes *Aex5* and *Aey6*, while *Hy2*, *Hy3*, *Hx1* and *Hy6* may have resulted from slippage in the replication of a related biparental gene. We found that both symmetric and asymmetric somatic hybridization could promote the emergence of novel alleles. We discussed the origination of allelic variation of HMW-GS genes in somatic hybridization, which might be the result from the response to genomic shock triggered by the merger and interaction of biparent genomes.

Keywords Agropyron · HMW-GS · Inheritance and variation · Somatic hybrid introgression lines · Triticum

Abbreviations

AFLP Amplification fragment length polymorphism
GISH Genome in situ hybridization
HMW-GS High-molecular weight glutenin subunit
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

The high-molecular weight glutenin subunits (HMW-GS) are a set of well-conserved endosperm proteins synthesized in the grain of wheat and related grasses (Lawrence and Shepherd 1981; Shewry et al. 2003). In hexaploid wheat, they are encoded by the *Glu-1* homoeoloci located on the long arms of chromosomes 1A, 1B and 1D, with each locus comprising a pair of tightly linked genes encoding the x-type (*Glu-1-1*) and the y-type (*Glu-1-2*) subunits (Lawrence and Shepherd 1981; Payne 1987). Qualitative and quantitative variation in the HMW-GS is associated with 45–70% of the variation in bread-making performance of European wheat, even though they only represent about 10% of grain protein (Branlard and Dardevet 1985; Payne et al. 1987, 1988). Because of their importance for wheat quality improvement, a substantial number of *Glu-1* genes have been cloned (Forde et al. 1985; Sugiyama et al. 1985; Thompson et al. 1985; Halford et al. 1987; Anderson and Greene 1989; Anderson et al. 1989; Halford et al. 1992). Sequence analysis of these genes has shown that each contains a long repetitive region, flanking two highly conserved terminal non-repetitive domains. The repetitive

X. Gao and S. W. Liu contributed equally to the work.

X. Gao · S. W. Liu · Q. Sun · G. M. Xia (✉)
The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Science, Shandong University, 250100 Jinan, People's Republic of China
e-mail: xiagm@sdu.edu.cn

region includes tripeptides, hexapeptides and nonapeptides, with the tripeptide motif restricted to the x-type subunits.

Although HMW-GS are clearly important for the determination of end-use quality, the number of high quality alleles is rather limited within the bread wheat gene pool. Thus, some effort has been made to transfer alleles from related species, using either a wide crossing (Zhou et al. 1995) or a somatic hybridization approach. Our focus has been to take advantage of the latter route. We have so far succeeded in fusing the protoplasts of the bread wheat cultivar Jinan 177 (JN177) with UV-irradiated protoplasts of tall wheatgrass (*Agropyron elongatum* (Host) Nevski [*Thinopyrum ponticum*]) (Liu SW et al. 2007; Liu H et al. 2009). We have also attempted symmetric somatic hybridization, in which the tall wheatgrass protoplasts were not UV irradiated. Regenerant plants of this latter protoplast fusion resembled the tall wheatgrass parent, but inherited several introgression segments from wheat (Cui et al. 2009). Selections CU and XI (each derived from a single fusion cell) were particularly fertile. Here, we have investigated whether any novel HMW-GS alleles are present in these somatic hybrid regenerants.

Materials and methods

Plant materials

The plant material used in these experiments consisted of the tall wheatgrass and bread wheat biparents of the somatic fusion, five R₃ (third generation following the regeneration of the primary somatic hybrid) lines R₃CU1, R₃CU2, R₃CU3, R₃XI1 and R₃XI2. Karyotypic analysis indicated that the chromosome number of the in vitro cultured tall wheatgrass cells ranged from 60 to 70, while about 80% of R₃CU1–R₃CU3 and R₃XI1–R₃XI2 cells carried 66–70 chromosomes. Cui et al. (2009) showed, by a combination of cytological and marker analyses, that the wheat chromosome segments were introgressed into *A. elongatum* chromosomes in the genomes of R₁–R₃ regenerants. Both JN177 and the introgression lines were grown in a greenhouse separated from other wheat cultivars to avoid uncontrolled outcrossing.

SDS-PAGE analysis of HMW-GS

The HMW-GS content of JN177 was obtained by SDS-PAGE analysis (Feng et al. 2004) of a crude protein extract of an embryo-less half grains, while those of tall wheatgrass and the introgression lines were obtained from

extracts of the whole seed, as described by Mackie et al. (1996).

Cloning and characterizing of HMW-GS genes

Genomic DNA was extracted from the introgression line seedlings using the CTAB method (Murray and Thompson 1980). As the HMW-GS genes are intron less, genomic DNA was used as a PCR template to amplify the entire coding region. A pair of degenerate primers (P1: 5'-ATGGCTAAGCGGc/tTa/gGTCTCTTTG and P2: 5'-CTACTACTGGCTa/gGCCGACAATGCG) was designed on the basis of published DNA sequences. P1 includes the HMW-GS start codon, and P2 includes the two conserved tandem stop codons. PCR amplification employed a high fidelity LA *Taq* polymerase (TaKaRa Biotechnology, Dalian, China) with a GC buffer provided for GC-rich template. The amplification profile consisted of a denaturation step (95°C/5 min), followed by 28 cycles of 94°C/40 s, 68°C/4 min, and ending with an extension step (72°C/7 min). The amplicon was recovered from a 1% agarose gel, cloned into the pMD18-T vector (TaKaRa Biotechnology, Dalian, China), and transformed into *E. coli* DH10B competent cells. Sequencing was performed commercially (Invitrogen, Shanghai, China). Both amplification and cloning were repeated at least three times to minimize the risk of amplification and/or sequencing errors. Sequence analyses were carried out using the MEGA software package v3.1 (Kumar et al. 2004) along with standard programs available from NCBI (<http://www.ncbi.nlm.nih.gov/Tools/>) and EBI (<http://www.ebi.ac.uk/Tools/sequence.html>).

Bacterial expression of HMW-GS sequences

To express the mature introgression line HMW-GS proteins in *E. coli*, two sets of PCR primers (PF/PR1 and PF/PR2) were designed to amplify the sequences while excluding their signal peptides, and at the same time introducing cloning sites. The sequence of PF was 5'-ACCCATATGGAAGGTGAGGCCTCT-3', that of PR1 was 5'-CTAGAATTCCTATCACTGGCTGGCCGA-3' (for *Hy4*, *Hy3*, *Hy7*, *Hy8*) and that of PR2 was 5'-CTAG AATTCCTATCACTGGCTAGCCGA-3' (for *Hy6*). PF contains an *NdeI* site and both PR1 and PR2 an *EcoRI* site. The amplicons were cloned into the expression vector pET-24a (Novagen, Shanghai, China), and transformed into *E. coli* BL21 (DE3) pLysS competent cells (Promega, Shanghai, China). Heterologous expression was induced using standard methods (Sambrook et al. 1989) and proteins were extracted by dissolving cells in SDS-PAGE sample buffer for SDS-PAGE analysis (Wan et al. 2002).

Results

The HMW-GS content of the biparents and the introgression lines

The HMW-GS content of JN177 is 1Bx7.1 + 1By9.1; 1Dx2.1 + 1Dy12.1, while that of the tall wheatgrass consists of nine distinct subunits. The HMW-GS composition of the five introgression lines R₃CU1–R₃CU3 and R₃XI1–R₃XI2 was overall very similar to that of the tall wheatgrass, although a small number of novel subunits could be identified (Fig. 1). A gel separation of the amplicons derived from each of the five introgression lines was shown in Fig. 2. After restriction enzyme digestion mapping and terminal DNA sequencing, we confirmed that at least 14 distinct sequences had been amplified from the introgression lines (designated as *Hx1–Hx6* and *Hy1–Hy8*, according to their type and length).

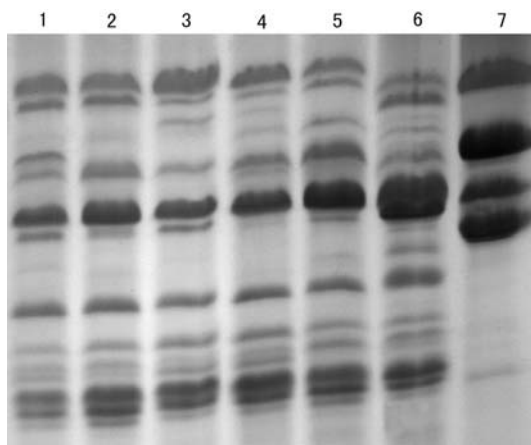


Fig. 1 HMW-GS profiles of some bread wheat/tall wheatgrass symmetric somatic hybridization derivatives and their parents. Lanes 1–5 R₃CU1–R₃CU3 and R₃XI1–R₃XI2 progeny, lane 6 tall wheatgrass, lane 7 JN177

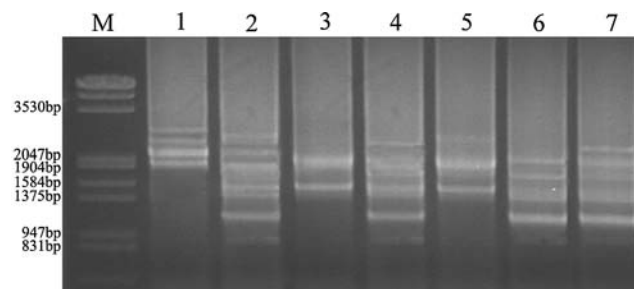


Fig. 2 PCR amplification of HMW-GS coding sequences from some bread wheat/tall wheatgrass symmetric somatic hybridization derivatives and their parents. M Lambda DNA digested by *EcoRI* + *HindIII*, lane 1 JN177, lane 2 tall wheatgrass, lanes 3–7 R₃CU1–R₃CU3 and R₃XI1–R₃XI2 hybrid spike lines, respectively

Expression of the HMW-GS alleles in bacterial cells

Five of the cloned sequences with intact ORFs were successfully expressed in *E. coli*, namely pET-Hy3, pET-Hy4 pET-Hy6, pET-Hy7 and pET-Hy8. The SDS-PAGE mobility of four of these (Hy4, Hy6, Hy3 and Hy8) was similar to that of equivalent subunits extracted from tall wheatgrass seeds, but there was no match between the proteins directed by pET-Hy7 and any tall wheatgrass seed-extracted protein (Fig. 3).

Characteristic of derived amino acid sequences of HMW-GS alleles

The deduced peptide sequences of the 14 HMW-GS genes shared the expected primary structure. Each consisted of a 21 residues signal peptide, a conserved N-terminal region, a central repetitive domain and a conserved C-terminal region. The N-terminal regions of five of the eight y-type subunits include 105 residues, while this length in Hy1, Hy4 and Hy5 was 104, 76 and 59 residues, respectively (Table 1). N-terminal regions of Hy1 lacked a glutamine residue when compared with Hy2, Hy3, Hy6, Hy7 and Hy8. This glutamine residue is also present in all the known x-type subunits. The conserved C-terminal regions of all the 14 subunits comprise 42 residues, and their central repetitive region included both hexapeptide and nonapeptide motifs; the six x-type subunits also contained the diagnostic GQQ tripeptide motif. Differences between these subunits and those already known in wheat lie mostly in single residue substitutions, and the insertion/deletion of repeat motifs in central repetitive region. The deduced peptide lengths of these subunits varied from 817 (Hx1) to 295

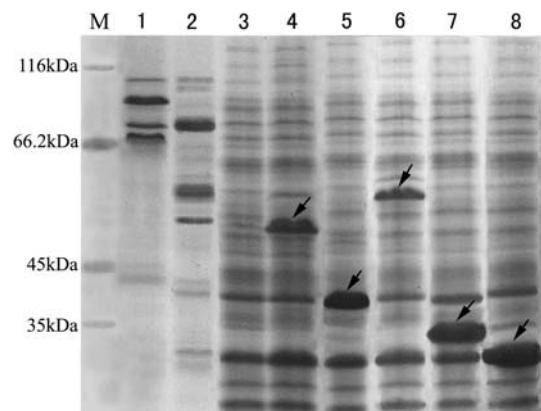


Fig. 3 SDS-PAGE analysis of heterologously expressed HMW-GS proteins. M protein molecular weight marker, lane 1 JN177, lane 2 tall wheatgrass, lane 3 bacteria harboring pET-*Hy4* without IPTG induction, lanes 4–8 expression of the modified sequences of *Hy4*, *Hy6*, *Hy3*, *Hy7* and *Hy8*. The HMW-GS gene-directed proteins induced by IPTG are indicated by arrows

Table 1 Sequence characteristics of the HMW-GS genes isolated from a set of bread wheat/tall wheatgrass symmetric somatic hybridization derivatives

Subunit	Accession number	Signal peptide Size (aa)	N-terminal region		Repetitive region		C-terminal region		Total	
			Size (aa)	Cys	Size (aa)	Cys	Size (aa)	Cys	Size (aa)	Cys
Hx1	GQ465208	21	86	3	668	0	42	1	817	4
Hx2	GQ465209	21	86	3	543	0	42	1	692	4
Hx3	GQ465210	21	86	3	536	0	42	1	685	4
Hx4	GQ465211	21	86	3	531	0	42	1	680	4
Hx5	GQ465212	21	86	3	516	0	42	1	665	4
Hx6	GQ465213	21	86	3	326	0	42	1	475	4
Hy1	GQ465214	21	104	5	442	1	42	1	609	7
Hy2	GQ465215	21	105	5	346	0	42	1	513	6
Hy3	GQ465216	21	105	5	342	0	42	1	509	6
Hy4	GQ465217	21	76	4	290	0	42	1	429	5
Hy5	GQ465218	21	59	3	288	0	42	1	410	4
Hy6	GQ465219	21	105	5	213	0	42	1	380	6
Hy7	GQ465220	21	105	5	128	0	42	1	295	6
Hy8	GQ465221	21	105	5	128	0	42	1	295	6

(Hy7 and Hy8) residues (Table 1). The Hy7 and Hy8 are also two of the smallest known HMW-GS.

Relationships between HMW-GS sequences

A phylogenetic tree was assembled from the alignment of the full-length nucleotide sequences of the 14 HMW-GS genes and the HMW-GS genes from JN177 and tall wheatgrass (Liu SW et al. 2007, 2008) (Fig. 4). As expected, the y-type genes were separated from the x-type ones. The eight y-type and the six x-type sequences each clustered into three clades. The *Hy1* sequence resembled that of tall wheatgrass *Aey6*, and was distantly related to the remaining seven y-type sequences, while *Hy6* was more similar to *Aey10* than to any of the other introgression line alleles. The other six y-type alleles of the introgression lines fell into three subgroups. *Hy2* and *Hy3* shared a close relationship with *Aey8*, while *Hy7* was similar to *Hy8*, as were *Hy4* and *Hy5*. Of the six x-type alleles, *Hx1* was similar to *Aex2*, *Hx5* to *Aex5* and quite closely related to *Hx2*, *Hx3* and *Hx4*, *Hx6* was an outlier within the x-type clade.

Discussion

Asymmetric somatic hybridization between bread wheat and UV-irradiated tall wheatgrass is known to generate wheat-like introgression lines (Xia et al. 2003; Wang et al. 2005), among which a deal of allelic variation for the HMW-GS genes has been identified (Liu SW et al. 2007; Liu H et al. 2009). Symmetric somatic hybridization involving the same biparents has produced fertile regenerants which more

resemble tall wheatgrass in phenotype, but whose genomes still contain some introgressed wheat segments (Cui et al. 2009). Therefore, we obtained a contrary introgression line of wheat/tall wheatgrass, which is favorable for exploring the variation of HMW-GS in different introgression lines via symmetric or asymmetric somatic hybridization.

The *Hx5* and *Hy1* sequences each differed by only a small number of single nucleotides from a tall wheatgrass sequence (*Aex5* and *Aey6*, respectively), and they had no close match with any of the HMW-GS sequences present in another parent JN177. Thus, it is likely that both were inherited from the tall wheatgrass parent, suffering some point mutation as a result of the somatic hybridization process. Similarly, *Hy2* and *Hy3* resembled *Aey8*, but for the presence of additional repeat motifs and a few single-nucleotide polymorphisms. *Hx1* and *Hy6* resembled *Aex2* and *Aey10*, respectively. When compared with *Aex2*, *Hx1* gained three additional repeats but lost one, while, compared to *Aey10*, *Hy6* gained one and lost two (Fig. 5). Possibly, therefore, these four *Glu-1* alleles may have derived via slippage of their corresponding parental gene during replication. The reason for why we have not found the origin of the other six novel HMW-GS sequences of the introgression lines might be that *A. elongatum* was a cross-pollinating species and there were plenty of the *Glu-1* alleles in *A. elongatum* and we have only obtained a limited number of *Glu-1* alleles from them (Liu SW et al. 2008).

The addition or deletion of repeat motifs is thought to be an effective source of variation (Wells 1996), while Anderson and Greene (1989) have proposed that the evolution of HMW-GS genes proceeds via a combination of single base changes, deletions or additions within a repeat, single

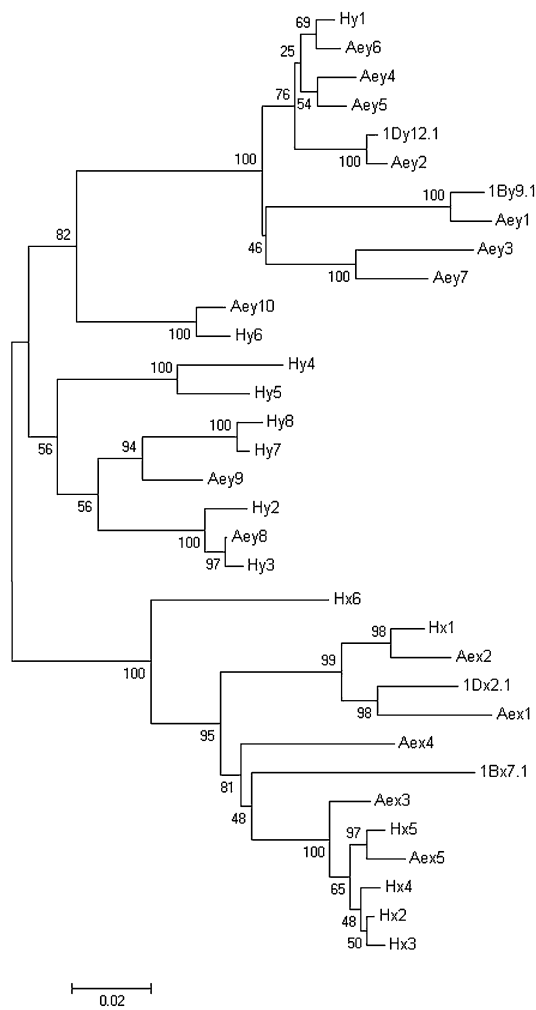


Fig. 4 Phylogenetic analysis of the HMW-GS sequences in some bread wheat/tall wheatgrass symmetric somatic hybridization derivatives and their parents. The phylogenetic tree was constructed according to the full-length DNA sequences using the MEGA software package v3.1. *Hy1–Hy8* and *Hx1–Hx6* came from the somatic hybridization derivatives; *Aey1–Aey10* and *Aex1–Aex5* came from tall wheatgrass; *1Bx7.1*, *1By9.1*, *1Dx2.1* and *1Dy12.1* came from JN177

repeat changes and deletions or duplications of blocks of repeats. The formation of some novel hybrid genes was inoculated with the mechanism mentioned by Anderson and Greene (1989). Thus, the forms of novel HMW-GS alleles generated in these introgression lines are consistent with that of naturally emerging ones, although the process of their formation appears to be accelerated by the somatic hybridization procedure.

Both the present symmetric hybridization experiments, as well as those based on the asymmetric hybridization (Liu SW et al. 2007; Liu H et al. 2009) have produced regenerants carrying a number of novel HMW-GS alleles. Although the regenerants from JN177 callus have also been shown to produce novel HMW-GS alleles, the somaclonal mutation rate is much lower (Feng et al. 2004). Although some of the

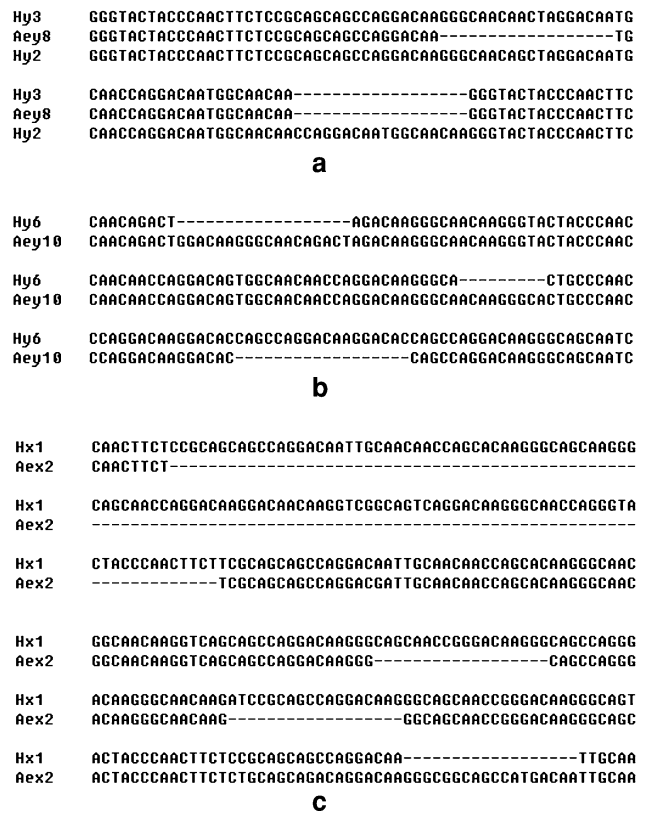


Fig. 5 Comparisons of the primary structure of HMW-GS sequences extracted from some bread wheat/tall wheatgrass symmetric somatic hybridization derivatives and their parents. **a** *Hy2* and *Hy3* versus *Aey8*, **b** *Hy6* versus *Aey10*, **c** *Hx1* versus *Aex2*

somatic hybridization-induced alleles may have arisen through somaclonal variation, it seems likely that many resulted from an interaction between the biparental genomes and/or the process of protoplast fusion itself; in the case of the asymmetric hybridization products, an additional source of variation is provided by the pre-fusion UV-irradiation treatment (Liu H et al. 2009). The analysis of certain newly synthesized allopolyploids has shown that when two genomes are united in a single nucleus, some instability ensues, which results in the elimination of genomic DNA sequences, the alteration of cytosine methylation patterns, and the reactivation of retrotransposons (Shaked et al. 2001; Ozkan et al. 2001; Madlung et al. 2002, 2005). Similar instability is, therefore, not unexpected in a somatic hybrid, and this has been demonstrated in wheat/tall wheatgrass combinations in the form of variation at microsatellite sequences, the elimination of DNA sequences, changes in the pattern of cytosine methylation and silencing or activation of homoeologous alleles (unpublished). The wide hybridization of different genomes might trigger a genomic shock that lead to these responses and it fit McClintock’s view about genomic shock response that “initiates a highly programmed sequence of events within the cell that serves to cushion the effect of the shock” (McClintock 1984). Therefore, the response to

genomic shock triggered by the merger and interaction of biparent genomes might be mainly responsible for the sequence variation in the introgression lines.

In conclusion, we have shown here that the variation in the HMW-GS sequences can be induced by symmetric as well as asymmetric somatic hybridization. It is possible that some of the novel alleles may make a positive contribution to the wheat end-use quality and is favorable to the investigation of genome variation and evolution.

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