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HMW and LMW glutenin alleles among putative tetraploid and hexaploid European spelt wheat (*Triticum spelta* L.) progenitors

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Abstract The allelic compositions of high- and low-molecular-weight subunits of glutenins (HMW-GS and LMW-GS) among European spelt (*Triticum spelta* L.) and related hexaploid and tetraploid *Triticum* species were investigated by one- and two-dimensional polyacrylamide-gel electrophoresis (PAGE) and capillary electrophoresis (CE). A total of seven novel glutenin alleles (designated *A1a*, B1d*, B1g*, B1f*, B1j*, D1a** at *Glu-1* and *A3h* at the *Glu-3* loci, respectively) in European spelt wheat were detected by SDS-PAGE, which were confirmed further by employing A-PAGE and CE methods. Particularly, two HMW-GS alleles, *Glu-B1d** coding the subunits 6.1 and 22.1, and *Glu-B1f** coding the subunits 13 and 22*, were found to occur in European spelt with frequencies of 32.34% and 5.11%, respectively. These two alleles were present in cultivated emmer (*Triticum dicoccum*), but they were not observed in bread wheat (*Triticum aestivum* L.). The allele *Glu-B1g** coding for 13* and 19* subunits found in spelt wheat was also detected in club wheat (*Triticum compactum* L.). Additionally, two alleles coding for LMW-GS, *Glu-A3h* and *Glu-B3d*, occurred with high frequencies in spelt, club and cultivated emmer wheat, whereas these were not found or present with very low frequencies in bread wheat. Our results strongly support the secondary origin hypothesis, namely European spelt wheat originated from hybridization between cultivated emmer and club wheat. This is also confirmed experimentally by the artificial synthesis of spelt through crossing between old European emmer wheat, *T. dicoccum* and club wheat, *T. compactum*.

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Introduction

Spelt (*Triticum spelta* L.) is one of the husked hexaploid wheats which possesses the same genomes as bread wheat (*Triticum aestivum* L.) and four other hexaploid wheats including club wheat (*T. aestivum* ssp. *compactum* (Host) Thell, shot wheat (*T. aestivum* ssp. *sphaerococcum* (Perc.), macha (*T. aestivum* ssp. *macha* Bowden) and *T. aestivum* ssp. *vavilovii* (Jakubz.). Although there are few differences among the subspecies, the morphological character of spelt is more evident, typically characterized by a narrow, lax and pyramidal spike with a brittle rachis and adherent glumes, generally long spike internodes and non-spherical seeds. These characters appear primitive compared to free-threshing bread wheat. Archaeological evidence showed that spelt was cultivated as early as the Stone Age in Europe and in the late Bronze Age (approximately 1,000 B.C.) it became the main cereal of central Europe (Andrews 1964). Several lines of evidence, including historical, linguistic and archaeological, indicate that extensive spelt cultivation had been restricted to central Europe (Flaksberger 1930; Bertsch 1943; Kema 1992; Nesbitt and Samuel 1996). The acreage of spelt wheat even exceeded those of bread wheat in the first decade of the 20th century (Kema 1992). Although spelt is now considered a minor crop, it has gained renewed interest in recent years because of its specific characters for stress tolerance and quality (Campbell 1997). Furthermore, spelt wheat cultivation is also encountered in Asia. Kuckuck discovered spelt landraces being grown at the high plateau of west-central Iran (Kuckuck and Schiemann 1957). In comparison to European spelt, Asian spelt showed very few anatomical differences. Also Dorofejev (1969) reported on spelt cultivation in Asia: Aserbaijan, Armenia, Nagorny Karabach and in Nachicevan.

Although numerous studies on the origin of European spelt wheat have been undertaken, its evolution is far from being resolved (Blatter et al. 2002). Different hypotheses have been postulated and the main discrepancy is whether European spelt is an ancestor or a derivation of tetrapoid or hexaploid *Triticum* species or hybrids between them. McFadden and Sears (1946) postulated that spelt was the ancestor of free-threshing hexaploid wheats originated from the hybridization between tetraploid wheat (*Triticum dicoccum*, genome: AABB, see Kuckuck 1982) and *Aegilops tauschii* (DD) having occurred very close to the area of contact between these two species. Subsequently, spelt was considered to be transmitted to Europe with the spread of agriculture (Andrews 1964; Zohary and Hopf 2000). However, there are no archaeological remnants of spelt wheat on this route from Asia to Europe. Therefore it is argued that European spelt may have a different, independent origin than Asian spelt. Bertsch (1943) and his father Bertsch (1950) postulated the hypothesis that European spelt was formed by secondary hybridization between club wheat (*T. aestivum* ssp. *compactum*) and emmer wheat (*T. dicoccum*) at the beginning of the Bronze age. Already Mathis (in Schiemann 1932) has found progenies of hybrids between *T. dicoccum* and *Triticum compactum* lines that resembled European spelt. MacKey (1966) reconstructed fully fertile spelta types among the offspring from crosses between *T. compactum* and *T. dicoccum*. Recently, Ohtsuka (1998) also obtained fertile *Triticum spelta* types in derivatives of *T. compactum* and *T. dicoccum* hybrids.

The high- and low-molecular-weight subunits of glutenins (HMW-GS and LMW-GS) are main storage proteins in wheat endosperm and are encoded by genes at the *Glu-1* and *Glu-3* loci, respectively. Variations at each *Glu* locus have been extensively studied in the past two decades, mainly due to their relationships with bread-making quality (Payne 1987). On the other hand, storage protein polymorphisms are also useful genetic markers for crop origin and evolutionary studies (Fernandez-Calvin and Orellana 1990).

In the present study, one- and two-dimensional polyacrylamide-gel electrophoresis (PAGE) and capillary electrophoresis (CE) were used to detect glutenin variations among European spelt, emmer, club and bread wheat in order to further investigate the origin of European spelt wheat. The objective was to search for specific HMW and LMW glutenin alleles among different tetraploid and hexaploid wheats, which could reveal the phylogenetic relationship among different *Triticum* species.

Materials and methods

Plant materials

A total of 235 spelt cultivars and lines (*T. spelta* L.) from Central Europe, 13 spelt landraces from Iran, 123 club wheat (*T. compactum*) lines, 184 cultivated emmer accessions (*T. dicoccum* Schrank), two macha wheat lines (*T. aestivum* ssp. *macha* Bowden) G1260 and G1497, two shot wheat lines (*T. aestivum* ssp. *sphaerococcum* Bowden) Kolandi and Cownpore and two accessions of *T. aestivum* ssp. *vavilovii* (No. 22 and No. 23) were studied. The materials were provided by genebanks of Braunschweig and Gatersleben, Germany.

The materials were planted in 2001–2002 and the typical characteristics of the different *Triticum* species were morphologically identified. Only typical club wheat lines were included in the analysis of glutenin composition. One hundred and fifty bread wheat cultivars mainly cultivated in Europe were also analyzed. In addition, Chinese spring, Hope, Hanno, Alidos and a further 18 bread wheat cultivars (Apostle, Brimstone, Longbow, Avalon, Beaver, Riband, Alpe 1, Floreal, Liocorno, Ilves, Nisu, Gemini, Timone, Ruso, Pandas, Pricama, Prinqual and Salmone), kindly provided by G Branlard, France, were used as standards for HMW and LMW glutenin subunit identification. In addition, seeds of F_1 and F_5 generations from the hybridization between the old European emmer wheat cultivar Hokudai (*T. dicoccum*) and club wheat No.44 (*T. compactum* var. *humboldtii*) described by Ohtsuka (1998) were also investigated.

Extraction of HMW and LMW glutenin subunits

An improved procedure for glutenin extraction based on Singh et al. (1991) was used. Gliadins of crushed individual kernels were first extracted and removed consecutively with 70% ethanol for 2 hours, and then with 0.25 ml of 55% (v/v) isopropanol three-times at 65 °C. Glutenins were then extracted from each kernel residue by first adding 0.1 ml of 50% (v/v) propanol, 0.08 M Tris-HCl, pH 8.0 (solution A), containing 1% (w/v) freshly added dithiothreitol (DTT). After incubation for 30 min at 65 °C, 0.1 ml of solution A containing 1.4% (v/v) freshly mixed 4-vinylpyridine (VP) was added. After shaking for 30 min at 65 °C, the samples were centrifuged for 5 min at 13,000 g; 0.1 ml of the supernatant was transferred into a new tube (the rest of the supernatant was kept aside for A-PAGE analysis) and 0.1 ml of solution A, consisting of 1% SDS, 0.08 M Tris-HCl, 40% glycerol and 0.001% bromophenol blue, was added. The tubes were shaken for 30 min at 65 °C, centrifuged for 5 min at 13,000 g and 0.02 ml of the supernatant was used for sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE).

For acidic polyacrylamide-gel electrophoresis (A-PAGE) analysis (Morel 1994), the remaining supernatant mentioned above was each transferred into new tubes and 0.5 ml of cold acetone was added for precipitating glutenin protein. The precipitate was collected after centrifugation for 15 min at 5 °C. Acetone was removed and glutenin was dried under room temperature. The precipitated glutenin was solubilized in 0.08-ml sample buffer consisting of 6 M urea, 30% glycerol and 25 mM acetic acid. After fully mixing and centrifuging for 10 min, 10-μl samples were used for A-PAGE analysis.

The sample preparation for CE analysis was based on the method of Sutton and Bietz (1997) and Bean and Lookhart (1998). Flour from a single kernel was first extracted four times with 50% propanol for removing monomeric gliadins, and then glutenins were reduced and extracted with 0.2 ml of 50% propanol + 1% DTT for 30 min at 65 °C with regular stirring. After centrifuging for 10 min at 13,000 g, HMW-GS was precipitated from glutenin extracts with the addition of acetone to a final concentration of 40%. Precipitated HMW-GS was re-dissolved in 0.2 ml of 25% acetonitrile (ACN) + 0.1% trifluoroacetic acid (w/v). All samples were filtered through 0.45-μm filters and used for capillary electrophoresis analysis within 24 h of extraction.

One- and two-dimensional polyacrylamide-gel electrophoresis

SDS-PAGE analysis of HMW and LMW glutenin subunits were performed on a Hoefer vertical electrophoresis unit. The gel

dimensions were 15 × 17 × 0.1 cm, which contained either 15 or 20 samples. Discontinuous electrophoresis was conducted with 10%, 12.5% or 15% running gel and 4% stacking gel. SDS-PAGE was carried out at a constant current of 10 mA per gel for 3 h, followed by 14 mA per gel until the tracking dye (bromophenol blue) had eluted from the bottom of the gel (about 15 h for the 10% gel, 18 h for the 12.5% gel and 22 h for the 15% gel). The gel was then stained in a solution of 0.1% PhasGel Blue R (Amersham Pharmacia Biotech AB), 10% ethanol and 12.5% trichloro-acetic acid (TCA) for 24 h, and de-stained with distilled water.

A-PAGE of HMW-GS was performed according to Yan et al. (1999) using the same electrophoretic apparatus described as above. Electrophoresis was carried out at a constant voltage 220 V for 10 min, followed by 500 V for 4–5 h at a constant temperature of 14 °C. The gel slabs were fixed in 10% TCA for 10 min, and then stained with 0.08% PhasGel Blue R, 10% ethanol and 10% TCA for 24 h, and de-stained with distilled water.

Two-dimensional A-PAGE × SDS-PAGE was carried out according to Redaelli et al. (1995) with some modifications. The second-dimensional electrophoresis was performed in the same apparatus as above. After the first-dimension A-PAGE analysis, the gels were cut into single strips and incubated for 30 min at room temperature in the equilibration solution containing 10% glycerol, 2% SDS and 0.0625 M Tris-HCl at pH 6.8. The equilibrated gel strips were placed on top of the second-dimension SDS-PAGE gel (12.5%) prepared as described above. Electrophoresis was performed at 14 mA for 1 h followed by 25 mA for 15 h at a constant temperature of 10 °C, and then gels were stained as described above.

Capillary electrophoresis (CE) of HMW glutenin subunits

The capillary electrophoretic separations of the HMW-GS were carried out using the BioFocus 3000 instrument based on Bean and Lookhart (1998). CE separations were performed at 40 °C and 12.5 kV using 0.1 M phosphate-glycine buffer, pH 2.5, containing 20% acetonitrile (ACN) and 0.05% hydroxypropyl-methycellulose (HPMC) with an uncoated fused-silica capillary of 27-cm separation length and 50-μm internal diameter. HMW glutenin subunits were detected by UV absorbance at 200 nm. All samples were injected at 10 kV for 8 s. After each separation, capillaries were rinsed with 1 M phosphoric acid for 2 min, and then with separation buffer for 2 min.

HMW and LMW glutenin-subunit identification and nomenclature

The identification of high- and low-molecular-weight subunits of glutenins and alleles was based on the classification of Payne and Lawrence (1983) and Jackson et al. (1996), respectively. In the present study, new LMW glutenin bands that were not previously classified by Jackson et al. (1996), were numbered according to their different relative mobilities. HMW-GS identification by CE was achieved through comparison of single and mixture samples employing standard cultivars and the results obtained from various gel electrophoresis.

Results

Detection of novel HMW and LMW glutenin alleles in European spelt wheat

HMW glutenin subunits of all materials studied were firstly separated and identified by 15% SDS-PAGE. The subunit compositions and their frequencies among the 235 European spelt cultivars and lines are listed in Table 1. Some typical HMW glutenin subunits are shown in Fig. 1. At the *Glu-A1* locus, the allele *a* coding for subunit 1 was found in 200 spelt cultivars and lines occurred with the highest frequency of 85.11%. A novel allele named *a** coding for subunit 2.1* located between subunit 1 and 2* was detected in two accessions, which was not found in previous investigations on spelt and common bread wheat. The *Glu-B1* locus exhibited the highest allelic

Table 1 Some specific HMW and LMW glutenin subunits and alleles of spelt wheat and their frequencies in comparison with club, emmer and common wheats

Glutenins	Subunits and alleles	Spelt wheat (235)		Club wheat (88 + 35)		Cultivated Emmer (184)		Bread wheat[a]
		No.	%	No.	%	No.	%	
HMW-GS								
Glu-A1	2.1* (a*)	2	0.85	5	5.68	9	4.89	–
	1 (a)	200	85.11	31	35.2	102	55.43	28% (1)
Glu-B1	6.1 + 22.1(d*)	76	32.34	–	–	13	7.07	–
	6.1 + null(j*)	3	1.82	5	5.68	8	4.35	–
	Null + 22.1(k*)	–	–	–	–	2	1.09	–
	13* + 19*(g*)	7	2.98	21	23.86	–	–	–
	13 + 22*(f*)	12	5.11	–	–	34	18.48	–
	13 + 16(f)	98	41.7	1	1.14	–	–	Rare (1)
	13(l)	–	–	–	–	2	1.09	–
	6 + 16(m)	–	–	–	–	4	2.17	–
	7 + 16(n)	–	–	–	–	5	2.72	–
Glu-D1	Null + 12(a*)	2	0.85	1	1.14	–	–	–
LMW-GS	3 + 6 + 7 + 9(I)	62	27.23	31	35.23	7	3.80	
	3 + 6 + 7 + 8(II)	40	17.02	16	18.18	18	5.40	
	3 + 5 + 6 + 7 + 8(III)	44	18.72	23	26.14	7	3.80	
	1 + 2 + 6 + 7 + 8(IV)	31	13.19	15	17.05	12	6.52	
	5 + 6 + 7 + 8(V)	28	11.91	11	12.50	12	6.52	
Glu-A3	6 + 9 (*h*)	149	63.40	63	71.59	33	17.93	–(2)
Glu-B3	3 (*d*)	182	74.45	55	62.50	41	22.28	4.08 (2)

[a] Payne and Lawrence (1983); Jackson et al. (1996)

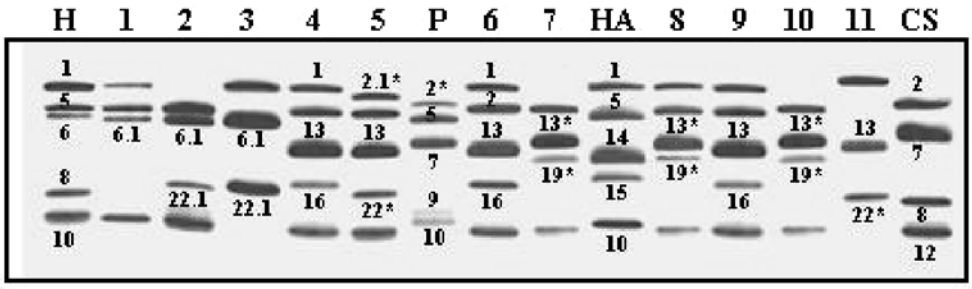

Fig. 1 The SDS-PAGE (15%) of some novel HMW glutenin subunits detected in central European spelt (*T. spelta* L.), club wheat (*T. compactum*) and cultivated emmer (*T. dicoccum*). (**1**) TRI4613/75 (spelt), (**2**) Hercule (spelt), (**3**) TRI16794/94 (*T. dicoccum*), (**4**) and (**9**) Bauländer Spelz (spelt), (**5**) 1094 (spelt), (**7**) Waggershauser Hohenheimer (spelt), (**8**) Renval (spelt), (**10**) NGB4792 (club), (**11**) PI94680 (*T. dicoccum*), (**H**) Hope, (**HA**) Hanno, (**CS**) Chinese Spring

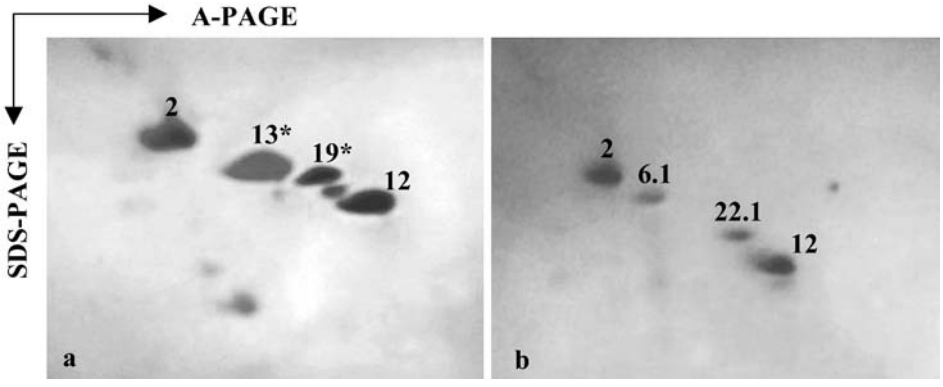

Fig. 2a, b Two-dimensional gel electrophoresis (A-PAGE × SDS-PAGE) of spelt wheat containing novel HMW glutenin subunits. (**a**) SP1 (N, 13* + 19*, 2 + 12), (**b**) Steiners Roter Tiroler (N, 6.1 + 22.1, 2 + 12)

variation among the three loci and two main alleles were identified. The first allele *f* coding for subunit pair 13 + 16 was present in spelt wheat with the highest frequency of 41.7%, but rare in bread wheat. The second allele designated *d** and coding for subunit pair 6.1 + 22.1 occurred in 32.34% of spelt cultivars and lines. This is a novel allele which was not found in a previous survey of spelt and bread wheat. As shown in Fig. 1, the electrophoretic mobility of subunit 6.1 was faster than that of subunit 6 while subunit 22.1 was situated between subunits 8 and 16. Further, two novel alleles designated *f** (13 + 22*) and *g** (13* + 19*) with 5.11% and 2.98%, respectively, were also detected in spelt wheat. The subunit 22* was situated between subunit 22.1 and 8, while subunits 13* and 19* flanked subunit 13, respectively. At the *Glu-D1* locus, allele *a* coding for the subunit pair 2 + 12 occurred with a frequency of 91.91%, and only 3.4% of the cultivars and lines possessed the allele *d* coding for the subunit 5 + 10. Two accessions were found to carry a new allele designated as *a** and determined subunit 12. In addition, the results from several *macha* and shot wheat accessions showed that only common subunits already documented in bread wheat were observed.

In order to confirm the presence of the above novel glutenin subunits, all spelt wheat accessions (each with 5–10 grains) containing new glutenin alleles were further separated and characterized by 12.5% and 10% SDS-PAGE, 10% A-PAGE, two-dimensional A-PAGE × SDS-PAGE and capillary electrophoresis. The results obtained from both 12.5% and 10% SDS gels were in agreement with those of the 15% gel, and each new subunit exhibited the same electrophoretic order as described above. Results of the A-PAGE analysis showed that subunits 13* and 19* moved apparently faster than 13 and 16, respectively, while subunit 22.1 exhibited a higher mobility than 8. The electrophoretic orders of the remaining subunits were the same as the results from SDS-PAGE. In comparison to the SDS-PAGE previous reports, the relative mobilities of some HMW-GS glutenins were changed when separated by A-PAGE, (Morel 1994; Yan et al. 2003). The two-dimensional electrophoretic patterns of four spelt-specific subunits are shown in Fig. 2, and both 13* + 19* and 6.1 + 22.1 subunits were well-separated and identified.

The capillary electrophoresis analysis of HMW subunits of glutenins showed that high-reproducible and high-resolution separation could be achieved by this method. In general, the separation of each sample can be completed in approximately 16 min, which is apparently faster than the 2–3-day period needed for the traditional gel-electrophoretic methods. All novel subunits detected by SDS-PAGE could be rapidly characterized by CE. The CE patterns of HMW glutenin subunits from two European spelt lines, Oberkulmer and Waggershauser Hohenheimer, are shown in Fig. 3a and b, respectively. It

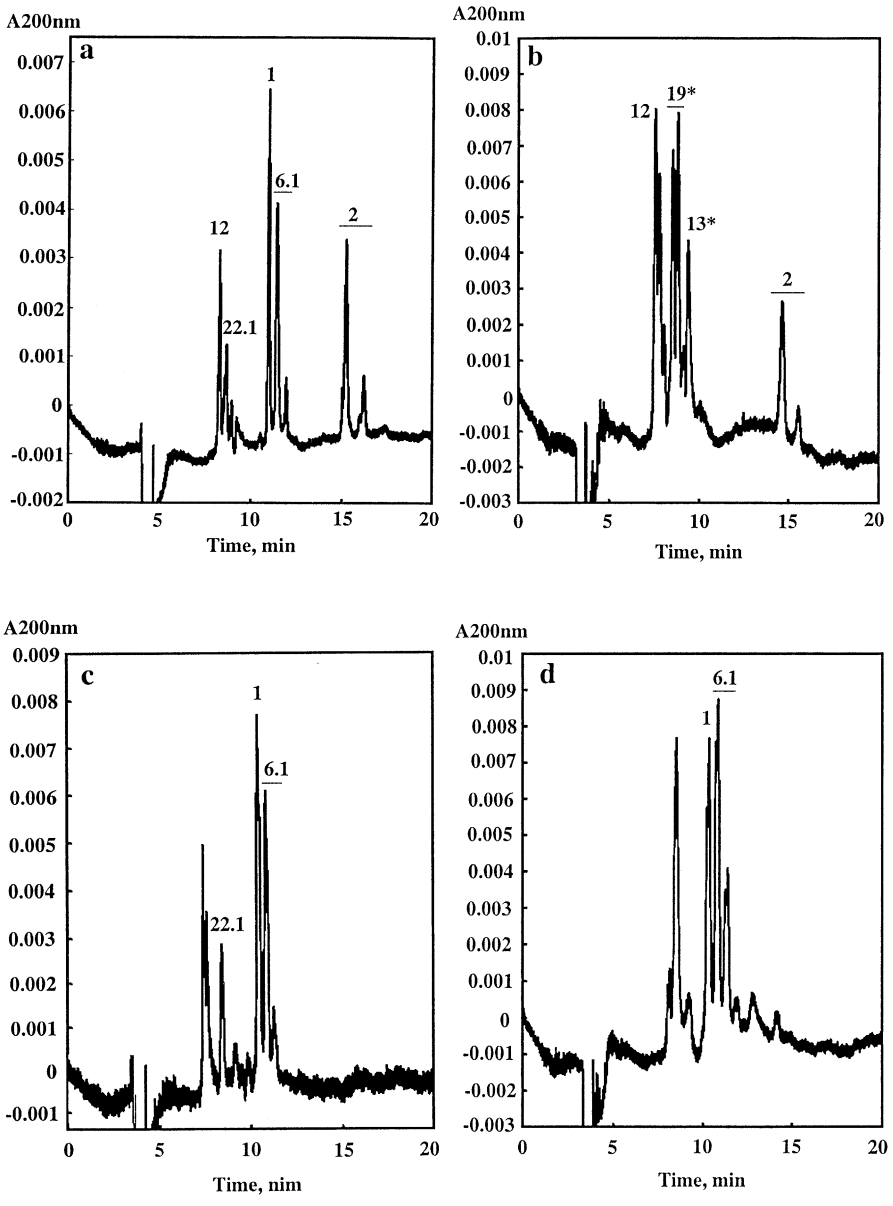

Fig. 3a–d Separation and characterization of HMW glutenin subunits in typical spelt, club and cultivated emmer accessions by capillary electrophoresis (CE) (**a**) Oberkulmer (spelt, 1, 6.1 + 22.1, 2 + 12). (**b**) Waggershauser Hohenheimer (spelt, N, 13* + 19*, 2 + 12). (**c**) TRI16794/94 (*T. dicoccum*, 1, 6.1 + 22.1). (**d**) 42074 (*T. dicoccum*, 1, 6.1 + null)

is evident that both the 6.1 + 22.1 and 13* + 19* subunits were well-separated. The relative migration orders of HMW-GS detected on the ascending migration time were as follows: 12 → 22.1 → 16 → 2.1* → 19* → 13* → 22* → 1 → 6.1 → 13 → 2. In particular, the subunits 19*, 22*, 6.1 and 2 were separated into two glutenin components, generally with a high peak accompanied by a minor peak, respectively. This was probably due to the higher resolution of CE or the different separation mechanism of HMW-GS. Similar results have been reported by Bean and Lookhart (1998).

The results from 13 Iranian landrace accessions revealed that all lines possessed HMW-GS already documented in common bread wheat, with the combinations N, 7 + 8, 2 + 12 and 2*, 7 + 8, 2 + 12 as the two main genotypes identified. The HMW-GS compositions of some typical European spelt wheats and important cultivars grown in Europe as well as the Iranian landrace spelt are listed in Table 2. It is apparent that three specific alleles at the *Glu-B1* locus (*d**, *g** and *f*) described above are present in both primitive spelt landraces and modern cultivated spelt cultivars. These primitive spelt accessions as well as many important modern cultivars including Altgold, Ostro, Hubel, Schwabenkorn, Rouguin, Hercule and Franckenkorn all possess the *Glu-B1d** allele. Another important cultivar, Bauländer Spelz, selected from a German landrace in 1953 possessed the most-encountered *Glu-B1f* allele while Renval and Waggershauser Hohenheimer carried the *Glu-B1g** allele. Although the frequency of this allele is low (2.98%) among the 235 accessions analyzed, it occurs with a high frequency in modern spelt cultivars. For example, among the 20 main spelt cultivars without common wheat introgression, four cultivars possess this allele with a

Table 2 Glutenin composition at *Glu-1* and *Glu-3* loci in primitive landraces, main cultivated spelt cultivars and lines of European and Iranian spelt accessions

Cultivars or lines	Origin	Year of release	References	*Glu-1*/*Glu-3* allelic composition		
				A1/A3	B1/B3	D1/D3
Oberkulmer	Switzerland	1948	Siedler et al. (1994)	a/h	d*/d	a/a
T. spelta var. *duhamelianum*	European primitive spelt	Prior to 1966	Ohtsuka (1998)	a/h	d*/d	a/a
Fuggers Babenhauser	Germany	Prior to 1900	BSA (2002)	a/h	d*/d	a/a
Vöglers Dinkel	Germany	1900	BSA (2002)	a/h	d*/d	a/a
Altgold	Switzerland	1952	Siedler et al. (1994)	a/h	d*/d	a/a
Ostro	Switzerland	1978	Siedler et al. (1994)	a/h	d*/d	a/a
Hubel	Switzerland	1992	Siedler et al. (1994)	a/a	d*/b	d/a
Rouquin	Belgium	1979	BSA (2002)	a/a	d*/d	d/a
Schwabenkorn	Germany	1988	BSA (2002)	a/h	d*/d	a/a
Hercule	Belgium	1982	Radic-Miehle et al. (1998)	c/h	d*/c	a/a
Franckenkorn	Germany	1995	BSA (2002)	a/a	d*/d	a/a
Renval	Belgium	1970		a/a	g*/d	a/a
Waggershauser Hohenheimer	Germany	1923		c/a	g*/b	a/a
Bauländer Spelz	Germany	1924	BSA (2002)	a/h	f/d	a/a
TA3377	Iranian land race	Detected: 1952	Kuckuck's collection	c/a	b/c	a/c
TA2687-1567	Iranian land race	Detected: 1952	Kuckuck's collection	b/a	b/g	a/a
TA2687-1570	Iranian land race	Detected: 1952	Kuckuck's collection	c/a	b/g	a/a

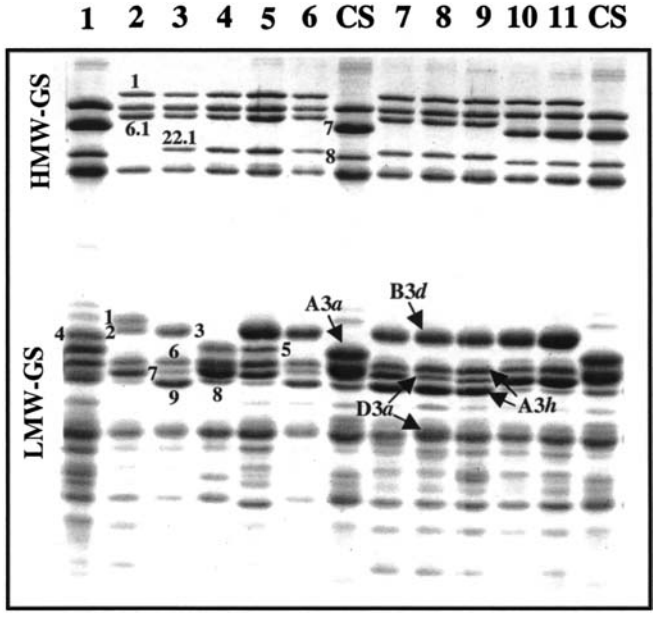

Fig. 4 SDS-PAGE (15%) of LMW glutenin subunits in some European spelt accessions. (**1**) TRI13352/82, (**2**) 3446, (**3**) 3445, (**4**) 3444, (**5**) 3443, (**6**) 3442, (**7**) 3440, (**8**) 3439, (**9**) 3438, (**10**) 3437, (**11**) 3436, (**CS**) Chinese Spring. LMW glutenin subunit bands with different relative mobilities are numerically named and the typical alleles at three *Glu-3* loci are *arrowed*

frequency of 20%. However, there are no distinctive specific alleles in the Iranian spelt landraces and all of the accessions analyzed share the same *Glu-B1b* allele with bread wheat.

Separation of LMW glutenin subunits showed that sharp protein patterns without gliadin contamination could be achieved by the sample preparation and SDS-PAGE methods used in this study (Fig. 4). Five main LMW-GS band combinations were detected in all European spelt accessions and their frequencies are listed in Table 1. The tentatively designated Type I with band numbers 3, 6, 7 and 9, corresponding to alleles *Glu-A3h, Glu-B3d* and *Glu-D3a* occurred with the highest frequency (27.23%), while the other four types were present in similar frequencies from 11.91% to 18.72% (Table 1). At the *Glu-B3* locus, the allele *Glu-B3d* (band 3) was found at a rather high frequency (77.45%) in spelt wheat, whereas it occurred rarely in bread wheat both in this study and the previous report of Jackson et al. (1996). A novel allele at the *Glu-A3* locus was detected, namely *h* (bands 6 and 9 in Fig. 4), which occurred in higher frequency (63.4%) in spelt wheat, but was not found in bread wheat. Most of the primitive spelt landraces and modern cultivars possess allele *h* at *Glu-A3, d* at *Glu-B3* and *a* at *Glu-D3*, respectively, while Iranian spelt landraces that were tested possessed alleles common to bread wheat (Table 1).

Comparison of common glutenin alleles in spelt, cultivated emmer (*T. dicoccum*) and club wheat (*T. compactum* L).

In order to understand the phylogenetic relationships of European spelt wheat, HMW and LMW glutenin allelic variations of related hexaploid and tetraploid species were compared. Noteworthy, some novel glutenin alleles detected in European spelt were found to occur simultaneously in club wheat and/or in cultivated emmer, whereas they did not appear in bread wheat and Iranian spelt landraces. As shown in Table 1, both *Glu-B1 d** (6.1 + 22.1) and *Glu-B1f** (13 + 22*) occurred in cultivated emmer (*T. dicoccum*) with frequencies of 7.07% and 18.24%, respectively, but they were not detected in club wheat. The allele *Glu-B1g** was present in club wheat with high frequency (23.86%), but was not detected in cultivated emmer. Allele *j** (6.1) was found in spelt, club

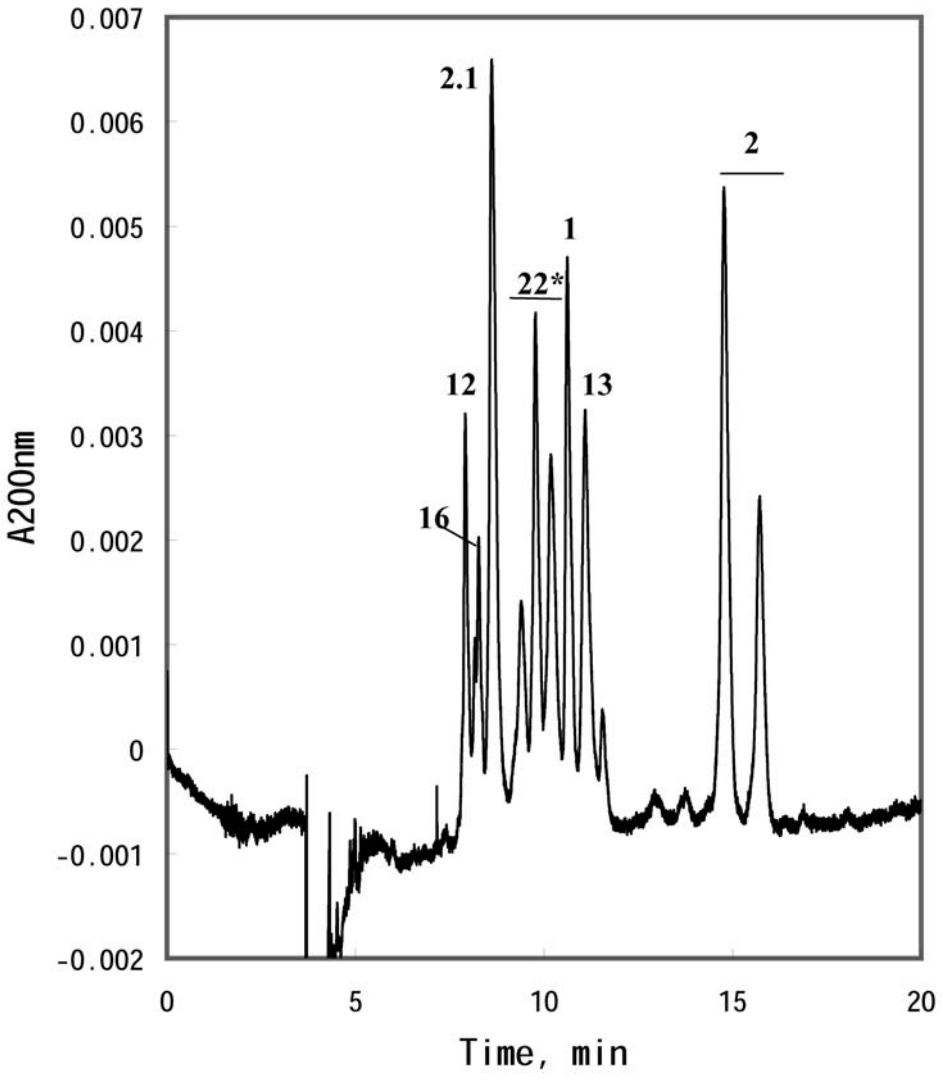

Fig. 5 Capillary electrophoresis of HMW-GS from mixture samples of club PI191580 (2.1*, 13 + 16, 2 + 12) and *T. dicoccum* 43531 (1, 13 + 22*) accessions. The CE conditions and HMW-GS identification were the same as described in Fig. 3

and emmer wheat, while allele *k** (22.1) was only observed in two emmer accessions. These alleles, however, were not detected in bread wheat. In addition, three other alleles at *Glu-B1*, namely *l* (13), *m* (6 + 16) and *n* (7 + 16), were uniquely detected in emmer wheat. Although allele *Glu-B1-f* occurred in spelt wheat with very high frequency, it was rarely found in club wheat and was not detected in cultivated emmer. At the *Glu-A1* locus, allele *a** (2.1*) was found to occur in both emmer and club wheat. This allele was also found in two spelt accessions, but was not detected in bread wheat. Allele *Glu-A1a* was commonly observed in spelt, club, emmer and bread wheat.

Several HMW glutenin alleles described above, including *A1a**, *B1d**, *B1g**, *B1f**, *B1f* and *B1j**, were clearly distinguishable by 10% and 12.5% SDS-PAGE, 10% A-PAGE and CE methods. The results of emmer and club wheat were well congruent with those of spelt wheat, obtained above. The CE patterns depicting novel HMW-GS from two accessions of emmer, TRI16794/94 (1, 6.1 + 22.1) and 42074 (1, 6.1), are shown in Fig. 3c and d, respectively, and all subunits were well separated and characterized. Generally emmer accessions showed one additional peak in about 7–8 min and a few minor bands with a little high mobilities were also observed. The minor proteins probably are not encoded by the known *Glu-1* loci, therefore further identification is needed. Figure 5 showed the CE pattern from mixture samples of club wheat PI191580 (2.1*, 13 + 16, 2 + 12) and emmer 43531 (1, 13 + 22*). Each subunit can easily be separated from the others and could be readily identified, thus confirming the identities of the various novel subunits.

The occurrence of LMW glutenin subunits indicated that European spelt wheat possessed similar frequencies of common alleles present in club and emmer wheats. Both the *Glu-A3h* and *Glu-B3d* alleles occurred in spelt, club and emmer wheats with high frequencies, but these alleles were rare in bread wheat (Table 1). Five LMW-GS combination types found in spelt also occurred with comparable frequencies in club wheat.

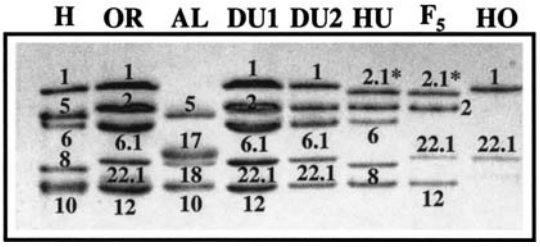

Fig. 6 HMW-GS composition of the F_5 hybrid between *T. compactum* var. *humboldtii* (**HU**) and *T. dicoccum* strain Hokudai (**HO**) cross in comparison with the primitive central European spelt wheat, *T. spelta* L. var. *duhamelianum* (**DU1** and **DU2**) and modern spelt cultivar Oberkulmer Rotkorn (**OR**). Bread wheat cultivars Hope (**H**) and Alidos (**AL**) were used as controls

Experimental reconstruction of European spelt wheat

The discovery and confirmation of several homogeneous HMW and LMW glutenin alleles among different tetraploid and hexaploid putative *Triticum* progenitor species strongly supports that the European spelt wheat originated from the hybridization between tetraploid emmer and hexaploid club wheat. Direct experimental reproduction of European spelt wheat had been carried out by crossing an old European emmer wheat *T. dicoccum* strain Hokudai and club wheat *T. compactum* var. *humboldii* strain no. 44 by Ohtsuka (1998). Original seeds from the different generations including the parents and the F_1 to F_5, populations were grown in Weihenstephan in 2002. It was observed that in the F_1 generation, the spike morphology was intermediate in size between the two parents possessing short awns. In the F_2 generation, segregation of several types were observed, with some plants resembling the old European *T. spelta* var. *duhamelianum* in ear and plant morphological characteristics. In the F_5 generation, some lines exhibited the uniform spike characteristics that were rather similar to the typical European spelt wheat.

Electropherograms of the parents showed that HMW-GS of the *T. dicoccum* and *T. compactum* parents were 1, null + 22.1 and 2.1*, 6 + 8, and 2 + 12, respectively. The selected F_5 synthesized spelt wheat showing morphological similarity to the control *T. spelta* var. *duhamelianum* possessed the 2.1*, null + 22.1, and 2 + 12 HMW-GS combination, which was found to be derived from the introgression of HMW-GS from both parents (Fig. 6).

Discussion

HMW-GS variations in European spelt wheat were previously investigated (Rodriguez-Quijano et al. 1990; Piergiovanni and Blanco 1999; Caballero et al. 2001) while studies on LMW-GS composition were not carried out. Previous reports also showed that some HMW-GS alleles and subunits, e.g. *A1a* (1), *B1f* (13 + 16) and *D1a* (2 + 12), occurred in rather high frequencies in spelt wheat. The most-encountered HMW-GS 13 + 16 in spelt, however, occurred rarely in club wheat and were not found in *T. dicoccum*, which is in agreement with previous investigations on club wheat (Rayfuse and Jones 1993) and on *T. dicoccum* (Vallega and Waines 1987). However, recent reports showed that a moderately high frequency (18.75%) of this allele occurred in club wheat (Ni et al. 2001). Several new alleles (Table 2) have been detected in spelt landraces grown in many countries. Oberkulmer was selected in 1948 from a Swiss landrace collected in the 1930s in Oberkulm (Blatter et al. 2002), and is involved in the parentage of many modern spelt cultivars (Siedler et al. 1994; Bertin et al. 2001). Fuggers Babenhauser and Vögelers Dinkel were selected from German landraces, and *T. aestivum* var. *duhamelianum* is an old European spelt that was already grown in the 19th century (Alefeld 1866). Novel alleles reported for the first time in the present study, especially *Glu B1d*, Glu-B1g** and *Glu B1f** that are typically present in European spelt wheat were not previously detected. This may be due to the fact that those materials studied were only restricted to Spanish accessions (Rodriguez-Quijano et al. 1990; Caballero et al. 2001). By far the efforts of systemic comparative analysis of glutenin alleles among different tetraploid and hexaploid *Triticum* species, and the phylogenic investigations of European spelt wheat, were limited.

In this study, electrophoretic patterns of novel alleles were further confirmed by different separation methods, including the recently developed capillary electrophoresis method with very high resolution. These alleles serve as reliable genetic markers and provide new biochemical evidence to reveal the origin and evolutionary pathway of European spelt wheat. Furthermore, these novel glutenin alleles do not occur in Iranian spelt landraces, which strongly support the independent origin, namely that European spelt was formed by hybridization between 4*x* emmer and 6*x* club wheat. Our results suggest that European spelt originated from several different hybridizations between emmer and club wheat in which the genotypes involved carried *Glu-A1a** and *A1a, Glu-B1d*, B1f, B1f** and*B1g**, and *Glu-A3h* and *B3d* alleles, respectively. However, the narrow genetic basis of European spelt revealed by both pedigree data and molecular markers, e.g. RFLP and microsatellites (Siedler et al. 1994; Bertin et al. 2001), suggested that the number of emmer and club wheat that were involved in the origin and evolution of European spelt was limited.

Our results showed that compositions of HMW and LMW glutenmin subunits of Iranian spelt were more similar to common bread wheat and no special alleles were found. Although the spelt accessions from Iran analyzed are limited, all materials tested in the present study belonged to primitive spelt landraces collected in Iran, thereby reflecting the real genetic background of Asian spelt. Furthermore, Iranian spelt is generally of the awn spike (Kuckuck and Schiemann 1957) while the ancient European spelt is typically of the awnless type (Ohtsuka 1998), e.g. Oberkulmer from a primitive landrace and *T. spelta* var. *duhamelianum*. Some Euro-

pean spelt wheats possess gliadin patterns and necrosis alleles that differ from Iranian spelt (Campbell 1997). To-date, extensive spelt cultivation from historical, linguistical and archaeological evidence had always been restricted to the central European area (Nesbitt and Samuel 1996). All these lines of evidence support that European spelt has originated in Europe independently from that of Iranian spelts. Archaeological findings showed that, since Neolithic times, club wheat, emmer wheat (*T. dicoccum*) and other related species, e.g. einkorn (*T. monococcum* L.) and free-threshing tetraploid wheats, were consistently present in Europe and by the late Neolithic period club wheat became the dominant cereal in the palafitte area (Andrews 1964). Therefore, the European spelt was the relatively recent strain and appeared later than club wheat, although it showed the primitive spike morphological characteristic. Furthermore, in combination with the recent research on HMW glutenin genes from modern and historical European spelt (Blatter et al. 2002) and the discovery of spelt-specific γ-gliadin genes (von Büren et al. 2000; von Büren 2001), all support the secondary origin hypothesis of European spelt wheat.

As known from previous reports (MacKey 1966; Ohtsuka 1998) on the experimental reproduction of spelt wheats, individual plants similar to typical European spelt could be obtained from the segregating populations from crosses between emmer and club wheats. On genetic considerations, these synthesised spelt should have the genes *C* (compact) from club wheat, as well as the *q* gene from *T. dicoccum*. The spikes of European spelt, however, do not possess the compact spike character. It is possible that the phenotypic expression of the *C* gene could be suppressed by other genes in the synthetic hexaploid genome, most likely the gene *q* (Ohtsuka 1998). The possibility of mutation or heterogenetic pairing still exist (MacKey 1966). Therefore, further studies are needed to resolve this problem.

According to the present results as well as other previous reports, it appears that Asian and European spelts have different origins and evolutionary pathways. The ancient spelt cultivation was still detected on the high plains of western Iran and genetic differences between Asian and European spelt were evidently observed, especially in HMW-GS in the present study and gliadin patterns as well as necrosis alleles and the genes controlling spike morphology (Campbell 1997; Luo et al. 2000).

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