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Distribution of the fertility-restoring gene *Rf3* in common and spelt wheat determined by an informative SNP marker

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Abstract Male sterility induced by the cytoplasm of *Triticum timopheevii* Zhuk. has shown potential for hybrid seed production in common wheat (*Triticum aestivum* L.). As hybrids produced by this method are often partially sterile, fertility restoration is crucial for implementing this technology in breeding practice. Several restorer genes were identified, of which *Rf3* is one of the most effective genes for achieving restoration. Previous studies located *Rf3* on chromosome 1B in common and spelt wheat. However, the distribution of *Rf3* in these taxa remained unclear. In the present study, we genetically mapped *Rf3* using a BC_1 population derived from CMS-Sperber and the restorer line Primepi (N = 193). After marker validation in four independent BC_1 populations and a diversity panel, we evaluated the distribution of *Rf3* in 524 common wheat and 30 European spelt genotypes. In the mapping population, the SNP marker *IWB72107* cosegregated with *Rf3*, whereas *IWB14060* was mapped 2.0 cM distal on chromosome 1BS. Surveying the linkage between *IWB72107* and *Rf3* in the four validation populations revealed map distances that ranged from 0.4 to 2.3 cM. Validation of *IWB72107* in the diversity panel showed that it is suitable for marker-assisted selection and related applications. Using this marker, we estimated that 8.8% of the common wheat lines and 66.7% of the spelt cultivars carried the restoring *Rf3* allele. We propose that *Rf3* explains the restoration capacity of a large proportion of European common wheat lines.

Keywords CMS · Fertility restoration · Hybrid wheat · Restorer gene

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Introduction

The shift from line to hybrid breeding in common wheat (*Triticum aestivum* L.) is currently one of the most debated issues in the wheat breeding community. Whereas the use of hybrids has been tremendously successful in crops such as maize, rice and rye (Crow 1998; Cheng et al. 2007; Geiger and Miedaner 2009), they remain of minor importance in wheat (Koekemoer et al. 2011). However, recent studies confirmed the fundamental assumption that hybrid wheat allows the exploitation of positive commercial heterosis for grain yield (Gowda et al. 2012; Longin et al. 2013). To make use of this advantage, several hybridisation technologies are available, of which cytoplasmic male sterility (CMS) and chemical hybridising agents are the most promising approaches (Whitford et al. 2013).

CMS in wheat was first reported by Kihara (1951) who obtained male-sterile plants by combining the nucleus of *T. aestivum* with the cytoplasm of *Aegilops caudata* L. A few years later, male sterility was also

observed when the cytoplasms of *Aegilops ovata* L. (Fukasawa 1953) and *Triticum timopheevii* Zhuk. (Wilson and Ross 1962) were used (Yen et al. 1969). *T. timopheevii* rapidly became the most common donor for sterility-inducing cytoplasms in wheat when it was reported that the two *Aegilops* cytoplasms showed adverse effects (Hayward 1975). Although many species have been used for the development of male-sterile wheat lines, the cytoplasm of *T. timopheevii* is considered to be the most reliable source to achieve male sterility (Koekemoer et al. 2011). In the present study, we exclusively refer to the CMS system based on this cytoplasm. Schmidt et al. (1962) and Wilson (1962) were the first to report fertility restoration of male-sterile wheat (Yen et al. 1969). Subsequently, restorer genes were found in many species including *T. timopheevii* (Livers 1964), *Triticum spelta* L. (Kihara and Tsunewaki 1967), *T. aestivum* (Oehler and Ingold 1966; Zeven 1967; Sinha et al. 2013), *Secale cereale* L. (Curtis and Lukaszewski 1993) and *Aegilops umbellulata* Zhuk. (Ma et al. 1995). The abovementioned genes were consecutively numbered from *Rf1* (Livers 1964) to *Rf8* (Sinha et al. 2013), with the exception of *Rfc3* and *Rfc4* (Curtis and Lukaszewski 1993). As these names were often used to refer to loci on different chromosomes, the nomenclature is not definite in the cases of *Rf2*, *Rf3*, *Rf4* and *Rf6* (Tahir and Tsunewaki 1969; Yen et al. 1969; Maan 1985; Ma et al. 1995; Kojima et al. 1997). In addition to restorer genes, environmental factors (Johnson et al. 1967) influence fertility restoration as along with epistatic effects of the genetic background (Maan et al. 1984). Fertility restoration became a crucial aspect in CMS hybrid wheat after it was discovered that the original sources for restorer genes resulted mostly in partially sterile hybrids (Keydel 1973; Hayward 1975). Johnson and Patterson (1977) showed that the combination of different sources of fertility restoration in one line led to an improved restoration capacity.

One of the most effective restorer genes is *Rf3*, which was discovered in European spelt on chromosome 1B (Tahir and Tsunewaki 1969), but may also control restoration capacity in some common wheat cultivars (Bahl and Maan 1973; Kučera 1982). Although *Rf3* was genetically mapped in several studies, there is no molecular marker suitable for the prediction of *Rf3* (Ma and Sorrells 1995; Kojima et al. 1997; Ahmed et al. 2001; Zhou et al. 2005). Whether the fertility-restoring allele of *Rf3* is fixed in the spelt population and how *Rf3* alleles are distributed in common wheat also remained unclear. To fill this knowledge gap, our objectives were to (1) genetically map the restorer gene *Rf3* in five biparental populations that involved restorer lines from common wheat and European spelt, and (2) evaluate the distribution of *Rf3* in German wheat breeding material and European spelt cultivars using a closely linked molecular marker.

Materials and methods

Plant materials

To identify molecular markers closely linked to the restorer gene *Rf3*, five BC_1 populations were developed. Initially, male-sterile lines were pollinated with the common wheat lines Primepi and PR143, and the spelt cultivars Badenkrone, Badenstern and Schwabenspelz, which were believed to carry fertility-restoring alleles at the *Rf3* locus (Tahir and Tsunewaki 1969; Bahl and Maan 1973; Patterson et al. 1996). Whereas Badenstern was combined with CMS-609-73, the other restorer lines were crossed with CMS-Sperber. The F_1 hybrids were subsequently backcrossed with the corresponding maintainer lines Sperber and 609-73. To determine the success of each cross, one spike per F_1 plant was bagged prior to anthesis and later examined for seed set. The BC_1 populations derived from Primepi, PR143, Badenkrone, Badenstern and Schwabenspelz comprised 193, 221, 290, 220 and 288 plants, respectively. The BC_1 seeds were sown into multi-pot trays. After vernalisation for 2 months at 5 °C in a growth chamber, seedlings were transferred to 13-cm pots (two plants per pot) and grown in a greenhouse under metal halide lamps at a temperature of 15–18 °C. Whereas the population CMS-Sperber/Primepi//Sperber was split into three subpopulations, with each being exposed to a different greenhouse environment, the other populations were grown in single environments.

To estimate the predictive ability of the markers linked to *Rf3*, we analysed a diversity panel comprising 29 common wheat and 30 European spelt lines. The accessions were classified based on their restoration capacity and references as described in detail in Table 2. The restoration capacity of the diversity panel was determined by testcrosses with CMS-Sperber and by assessing the seed set of the resulting F_1 plants. Common wheat accessions reported to carry the

fertility-restoring *Rf3* allele were used as positive controls. Common wheat lines served as negative controls either if they were known to possess no restoring allele on chromosome 1B or if they were not able to restore fertility in testcrosses. To expand the diversity panel, we determined the restoration capacity of 30 spelt cultivars. As fertility restoration in *T. spelta* var. *duhamelianum* is controlled exclusively by *Rf3* (Tahir and Tsunewaki 1969; Kojima et al. 1997), we used accessions of this taxon as positive controls if they were able to restore fertility. Spelt accessions showing no restoration capacity in testcrosses served as negative controls. Fertility restoring spelt lines that might belong to other varieties than var. *duhamelianum* and that were not shown to carry the restoring *Rf3* allele by linkage mapping were excluded from the diversity panel.

The distribution of *Rf3* alleles in common wheat and spelt accessions was estimated using 524 German winter wheat breeding lines and 30 European spelt cultivars, of which the latter were also analysed for the diversity panel. To verify whether *Rf3* genotypes of the spelt cultivars depend on their relationship to common wheat, we analysed them together with a set of 368 German common wheat cultivars.

The French winter wheat cultivar Primepi and the Belgian winter wheat cultivars Minister and Professeur Marchal were obtained from the gene bank of IPK Gatersleben, Germany. The winter wheat lines PR143 and PR189 were provided by the National Small Grain Collection, ID, USA. Maintainer lines Navojoa and Vorobey were released by CIMMYT, Mexico. The restorer lines R1 and R3 were provided by Sejet Plant Breeding, Denmark. The 524 common wheat breeding lines used to determine the distribution of *Rf3* were provided by the German breeding companies Saatzucht Bauer GmbH & Co. KG, Saatzucht Josef Breun GmbH & Co. KG, Limagrain GmbH, SECOBRA Saatzucht GmbH, Saatzucht Streng-Engelen GmbH & Co. KG and Lantmännen SW Seed GmbH. The remaining common wheat and spelt lines were in stock at the germplasm collection of the Bavarian State Research Center for Agriculture.

Assessment of fertility restoration

As a measure for the restoration of male fertility, we determined the seed set of isolated spikes. Before anthesis, one to four emerging spikes of each plant were isolated using glassine bags. After ripening, the bagged spikes were harvested. For each of these spikes, the numbers of spikelets and kernels were recorded. The seed set of a plant was defined as the number of kernels divided by the number of spikelets, averaged over the isolated spikes. A plant was considered male sterile if it contained no seeds, whereas it was considered male fertile if it contained at least one seed. The ratios of fertile and sterile plants of the mapping populations were compared to the expected segregation pattern using Pearson's χ^2 test ($\alpha = 0.05$). Modalities of the seed set distributions were analysed using Hartigan's dip test ($\alpha = 0.05$). Statistical analysis of the seed set was performed using R (R Core Team 2015).

Genotyping and linkage mapping

Total genomic DNA was extracted from young leaf tissue according to the protocol described by Plaschke et al. (1995). Initially, *Rf3* was mapped using the BC_1 population CMS-Sperber/Primepi//Sperber. The 193 individuals of the population were genotyped with the simple sequence repeat (SSR) markers *Xbarc8*, *Xbarc128*, *Xgwm264*, *Xwmc406* and *Xwmc798*, which are located on chromosome 1BS (Somers et al. 2004). Primer sequences and amplification conditions were obtained from the GrainGenes database (http://wheat.pw.usda.gov). To increase the marker density in the genomic region of *Rf3*, we selected individuals that showed recombination for the SSR loci flanking the restorer gene. Recombinant individuals were genotyped by TraitGenetics GmbH (Gatersleben, Germany) using an Illumina® Infinium® 15 k single nucleotide polymorphism (SNP) array based on the 90 k SNP array described by Wang et al. (2014). SNPs linked to *Rf3* in this population were converted to cleaved amplified polymorphic sequence (CAPS) markers by aligning multiple DNA sequences obtained from The Triticeae Toolbox (https://triticeaetoolbox.org), CerealsDB (http://www.cerealsdb.uk.net), Unité de Recherche Génomique Info (https://urgi.versailles.inra.fr), GrainGenes (http://wheat.pw.usda.gov) and KOMUGI (http://shigen.nig.ac.jp) using the Clustal Omega program (http://www.ebi.ac.uk). Based on this sequence alignment, we designed polymerase chain reaction (PCR) primers using Primer3web (http://primer3.ut.ee). Restriction enzymes were chosen with the analysis tool SNP2CAPS (IPK, Gatersleben, Germany). The CAPS markers were subsequently validated by genotyping the population CMS-

Sperber/Primepi//Sperber. One of the converted markers was used to genotype a sample of each of the populations derived from PR143 (N = 170), Badenkrone (N = 284), Badenstern (N = 87) and Schwabenspelz (N = 87). Products of SSR and CAPS markers were resolved on 5% polyacrylamide gels. For the construction of linkage maps, we used JoinMap® 4.0 (Kyazma B.V., Wageningen, The Netherlands). Linkage groups were established using an independence logarithm of the odds (LOD) value of ≥6.0. Regression mapping was performed using the Kosambi mapping function (Kosambi 1944).

Distribution of *Rf3* in common wheat and European spelt

To estimate the distribution of *Rf3* in common wheat, a sample of 524 current German breeding lines was genotyped with the Illumina® Infinium® 15 k SNP array. The frequency of *Rf3* genotypes was estimated based on the SNP marker linked with *Rf3*. The distribution of *Rf3* in common wheat was evaluated by principal coordinate analysis (PCoA) using modified Roger's distances between the breeding lines (Wright 1978). To verify whether *Rf3* genotypes were homogeneously distributed within the population, we calculated Pearson's correlation coefficients between binary *Rf3* marker genotypes and the principal coordinates. Significance of the correlations was tested using t tests (α = 0.05).

The distribution of *Rf3* was additionally estimated in the 30 spelt cultivars also used for the diversity panel. The spelt cultivars were genotyped together with 368 German common wheat cultivars using 33 SSR markers evenly distributed across the genome. We performed PCoA for the spelt lines as well as for the spelt lines together with the 368 wheat cultivars. The distribution of *Rf3* in spelt was examined as described for common wheat. To test for a possible relationship between *Rf3* genotype classes of the spelt lines and their kinship to common wheat, we calculated the mean genetic distance to common wheat for each spelt cultivar. Mean distances of spelt cultivars with the restoring *Rf3* allele were compared to mean distances of spelt cultivars without the restoring *Rf3* allele using t tests (α = 0.05). PCoA was performed within the R environment (R Core Team 2015) using the R package "APE" (Paradis et al. 2004).

Results

Seed set of the mapping populations

Segregation into fertile and sterile plants conformed with the expected 1:1 ratios in all populations, indicating a monogenic inheritance of fertility restoration (Table 1). This ratio was also observed for the three subpopulations of CMS-Sperber/Primepi//Sperber ($P_{\chi}^2 \geq 0.35$). The mean seed sets of the fertile plants ranged from 0.54 to 1.57 seeds per spikelet, with the lowest and highest seed set in CMS-609-73/Badenstern//609-73 and CMS-Sperber/Primepi//Sperber, respectively. At 0.57 seeds per spikelet, the standard deviation of the seed set was higher in CMS-Sperber/Primepi//Sperber than in other populations. Seed set distributions of the fertile plants are shown in Fig. S1. Hartigan's dip test suggested a unimodal seed set distribution for the fertile plants in each population.

Genotyping and linkage mapping

Using the categorical fertility phenotypes (completely sterile or fertile) and SSR genotypes of the BC_1 population CMS-Sperber/Primepi//Sperber, we constructed a partial linkage map of chromosome 1BS. The map comprised a single linkage group spanning 33.7 centimorgans (cM). *Rf3* was flanked by the SSR loci *Xbarc128* and *Xwmc406*, located 7.2 cM distal and 14.5 cM proximal to *Rf3*, respectively. To enrich this genomic region with SNP markers, we identified 40 plants that were recombinant between the flanking SSR loci. The selected individuals were genotyped with the 15 k SNP array. After removing markers with >10% missing alleles (N = 625) or a minor allele frequency <10% (N = 8705) as well as monomorphic markers (N = 883), 2793 SNPs remained for linkage mapping. We identified three normally inherited SNP markers closely linked to *Rf3*, namely *IWB14060*, *IWB72107* and *IWB73447*. The SNPs *IWB14060* and *IWB72107* were used to develop CAPS assays, designated *CAPS_IWB14060* and *CAPS_IWB72107*, respectively (Table S1). The development of a CAPS assay for *IWB73447* was not successful because it did not affect the recognition site for a restriction enzyme. The CAPS markers were validated by genotyping the population CMS-Sperber/Primepi//Sperber, and the original microarray-based SNP genotypes were reproduced. This validation also confirmed the assumed SNP

Table 1 Mean seed sets of fertile plants and segregation of restoration capacity for five mapping populations

BC_1 population	N	Mean	$N_{fertile}/N_{sterile}$	P_{χ}^{2}	$P_{Hartigan}$
CMS-Sperber/Primepi//Sperber	193	1.57 ± 0.57	104:89	0.28	0.57
CMS-Sperber/PR143//Sperber	221	0.91 ± 0.37	117:104	0.38	0.39
CMS-Sperber/Badenkrone//Sperber	290	1.02 ± 0.37	132:158	0.13	0.99
CMS-609-73/Badenstern//609–73	220	0.54 ± 0.34	104:116	0.42	0.80
CMS-Sperber/Schwabenspelz//Sperber	288	0.82 ± 0.35	151:137	0.41	0.99
Combined[a]	1212		608:604	0.23	

Segregation patterns were compared to a 1:1 ratio using χ^2 tests. Seed set distributions of fertile plants were analysed by Hartigan's dip test

[a] χ^2 test for homogeneity of fertility restoration across the BC_1 populations

genotypes of the non-recombinant plants that were not genotyped with the SNP array. The consensus map comprising five SSR and two SNP genotypes of all 193 individuals spanned 33.6 cM (Fig. 1). Whereas *IWB72107* cosegregated with *Rf3*, *IWB14060* was mapped 2.0 cM distal to *Rf3*. Using the CAPS assay *CAPS_IWB72107*, we mapped *Rf3* in the four populations that involved PR143, Badenkrone, Badenstern and Schwabenspelz. In these populations, *CAPS_IWB72107* was mapped 0.6 (1 recombinant), 0.4 (1 recombinant), 2.3 (2 recombinants) and 1.2 cM (1 recombinant) from *Rf3*. There were no instances of deviation from the expected genotype frequencies (Table S2). Map distances between *CAPS_IWB72107* and *Rf3* indicated that fertility restoration was controlled by the same locus, namely *Rf3*, in all mapping populations.

Predictive ability of *CAPS_IWB72107*

The CAPS assay *CAPS_IWB72107* based on the SNP *IWB72107* was further validated in a diversity panel comprising common wheat and European spelt accessions (Table 2). The marker genotype of Primepi was set as a reference for the prediction of the *Rf3* allele associated with fertility restoration. Using this marker, we predicted the correct *Rf3* allele in all 29 common wheat accessions. Analysing the restoration capacity of the spelt lines revealed that 20 of 30 spelt accessions restored fertility in testcrosses with CMS-Sperber. The five restoring spelt cultivars Ceralio, Tauro, Titan, Zollernspelz and Züricher Oberländer Rotkorn were excluded from the diversity panel as there was no clear record that they belong to the *T. spelta* var. *duhamelianum* taxon. *CAPS_IWB72107* predicted the correct *Rf3* allele in 23 of the remaining 25 spelt accessions. Only the marker genotypes of Bauländer Spelz and Grey did not correspond to their inability to restore fertility. *CAPS_IWB72107* produced the two alleles that were previously observed in the mapping populations as well as null alleles for the negative controls 444-74, 539-74, 563-76, R1, R3 and Samir and Sirino (Fig. S2). These results indicate that the SNP *IWB72107* is suitable for marker-assisted selection and related applications.

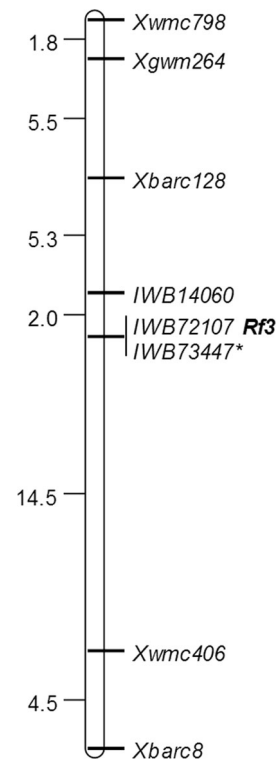

Fig. 1 Linkage map of chromosome 1BS in the mapping population CMS-Sperber/Primepi//Sperber. Map distances are in cM. The *asterisk* indicates the position of *IWB73447* based on 40 individuals

Table 2 *Rf3* alleles (+ fertility restoring allele; − non-restoring allele) and observed marker alleles for *CAPS_IWB72107* across the lines of the diversity panel

Accession	Taxon	*Rf3*	*CAPS_IWB72107*	Reference
Minister	*Triticum aestivum*	+	A	Kučera (1982)
PR143	*T. aestivum*	+[a]	A	Patterson et al. (1996); this study
Primepi	*T. aestivum*	+[a]	A	Bahl and Maan (1973); this study
Professeur Marchal	*T. aestivum*	+	A	Kučera (1982)
Alkor	*Triticum spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Altgold	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Badenkrone	*T. spelta*	+[a]	A	This study
Badenstern	*T. spelta*	+[a]	A	This study
Cosmos	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Divimar	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Ebners Rotkorn	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Epanis	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Holstenkorn	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Ostro	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Poeme	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Renval	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Rouquin	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Schwabenkorn	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969)
Schwabenspelz	*T. spelta* var. *duhamelianum*	+[a]	A	Tahir and Tsunewaki (1969); this study
10-77 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
1-78 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
107-77 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
31-77 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
406-76 (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication
435-76 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
441-78 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
444-74 (maintainer)	*T. aestivum*	−	Null	Keydel personal communication
463-77 (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication
50-74 (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication
539-74 (maintainer)	*T. aestivum*	−	Null	Keydel personal communication
563-76 (maintainer)	*T. aestivum*	−[b]	Null	Keydel personal communication
609-73 (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication
629-77 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
82-77 (maintainer)	*T. aestivum*	−	G	Keydel personal communication
Bert (maintainer)	*T. aestivum*	−	G	Keydel personal communication
Granit (maintainer)	*T. aestivum*	−	G	Keydel personal communication
Mission (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication
Navojoa (maintainer)	*T. aestivum*	−	G	Bonnett personal communication
PR189	*T. aestivum*	−[a]	G	Patterson et al. (1996)
R1	*T. aestivum*	−	Null	Bahl and Maan (1973)
R3	*T. aestivum*	−[a]	Null	Bahl and Maan (1973)
Severin (maintainer)	*T. aestivum*	−	G	Keydel personal communication
Sperber (maintainer)	*T. aestivum*	−[b]	G	Keydel personal communication

Table 2 (continued)

Accession	Taxon	*Rf3*	*CAPS_IWB72107*	Reference
Vorobey (maintainer)	*T. aestivum*	–	G	Bonnett personal communication
Albin	*T. spelta*	–[b]	G	
Badengold	*T. spelta*	–[b]	G	
Bauländer Spelz	*T. spelta* var. *duhamelianum*	–[b]	A	
Filderweiss	*T. spelta*	–[b]	G	
Franckenkorn	*T. spelta* var. *duhamelianum*	–[b]	G	
Grey	*T. spelta*	–[b]	A	
Hercule	*T. spelta* var. *album*	–[b]	G	
Oberkulmer Rotkorn	*T. spelta* var. *duhamelianum*	–[b]	G	
Samir	*T. spelta*	–[b]	Null	
Sirino	*T. spelta*	–[b]	Null	

[a] Restored fertility in the testcross with CMS-Sperber

[b] Did not restore fertility in the testcross with CMS-Sperber

Distribution of *Rf3* alleles in common wheat and European spelt

The frequency of *Rf3* alleles in common wheat was estimated using the *IWB72107* genotypes of 524 current German breeding lines; 8.8% of the lines carried the SNP allele associated with fertility restoration. Analysis of the population structure with PCoA revealed that *IWB72107* alleles were evenly distributed along the first four principal coordinates ($P \geq 0.11$), jointly explaining 13.5% of the genotypic variation, thereby indicating a homogeneous distribution of *IWB72107*, and thus *Rf3* alleles in this common wheat population. The first two principal coordinates are depicted in Fig. 2.

The *CAPS_IWB72107* genotypes and the restoration capacity of 30 spelt cultivars indicated that 20 cultivars (66.7%) carried the fertility-restoring allele at the *Rf3* locus. The five fertility restoring spelt accessions that were discarded from the diversity panel were included here, since the marker *CAPS_IWB72107* predicted the restoring *Rf3* allele for all of them. As the accessions Bauländer Spelz and Grey could not restore fertility in testcrosses, they were classified as carriers of the non-restoring *Rf3* allele. The *Rf3* genotypes of the spelt cultivars were not significantly correlated to any of the first four principal coordinates ($P \geq 0.06$), which together explained 40.8% of the genotypic variation, suggesting that *Rf3* alleles are homogeneously distributed within this spelt population (Fig. S3). Mean genetic distances between fertility-restoring spelt cultivars and common wheat were not significantly different to the distances observed for the spelt cultivars without the restoring *Rf3* allele ($P = 0.052$), suggesting that *Rf3* genotypes of spelt cultivars were not dependent on their kinship to common wheat (Fig. 3).

Discussion

We genetically mapped the fertility-restoring gene *Rf3* using five populations derived from restorer lines of common wheat and European spelt. The segregation

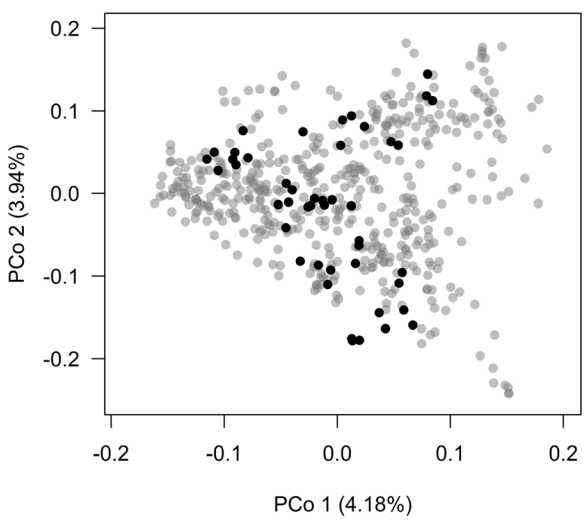

Fig. 2 Principal coordinate analysis of German common wheat breeding lines. *Black dots* represent lines with the *IWB72107* allele associated with the fertility-restoring allele of *Rf3*

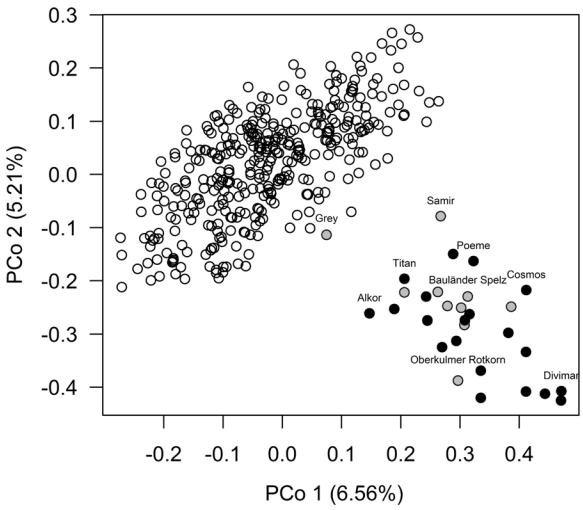

Fig. 3 Principal coordinate analysis of 30 European spelt cultivars together with 368 common wheat lines. Spelt accessions were classified as carriers of the restoring (*black dots*) or non-restoring/null (*grey dots*) allele at the *Rf3* locus. Common wheat lines are depicted in *white*

of fertility restoration into fertile and sterile plants indicated monogenic inheritance of fertility restoration in all five populations. This is in agreement with the unimodal distributions we observed for the seed set of the fertile plants. Linkage mapping indicated that restoration was controlled exclusively by *Rf3*, located on chromosome 1BS. Since each population was exposed to a different environment, we could not compare the restoration capacities of the five restorer lines. In CMS-Sperber/Primepi//Sperber, there was a high standard deviation of the seed set compared to the other populations. This can be explained by the fact that each of the three subpopulations was exposed to a different environment, which had a significant effect on the seed set of the fertile plants (data not shown). The monogenic inheritance of fertility restoration we observed in CMS-Sperber/Primepi//Sperber is in accordance with the findings of Ingold (1968), who reported a single restorer gene in Primepi. However, Bahl and Maan (1973) located two restorer genes in Primepi by monosomic analysis: one with higher expressivity located on chromosome 1BS (*Rf3*), and one with lower expressivity located on chromosome 5D (unnamed). The hypothesis of two restorer genes was confirmed by Miller et al. (1974), who observed a 9:6:1 ratio of fertile, partially fertile and sterile individuals, respectively, in a segregating F_2 population involving Primepi. This discrepancy might be due to environmental effects or the genetic background of the CMS lines influencing the expressivity of the restorer gene on chromosome 5D. An alternative explanation may be that different Primepi accessions are polymorphic at this locus. Previous studies have not as yet described the genes controlling the restoration capacity of PR143. PR143 descends from a cross between R3 and Monon/Primepi and was bred by recurrent selection for an optimised restoration capacity (Patterson et al. 1996). According to its pedigree, PR143 could possibly carry alleles for fertility restoration at the *Rf1*, *Rf2* and *Rf3* loci, as well as the one on chromosome 5D. In contrast, we observed that *Rf3* was the only gene controlling fertility restoration in the population CMS-Sperber/PR143//Sperber. Monogenic inheritance of fertility restoration controlled by *Rf3* was also observed in the three populations derived from the European spelt cultivars Badenkrone, Badenstern and Schwabenspelz. This is in accordance with the observations of Tahir and Tsunewaki (1969) and Kojima et al. (1997), who observed that fertility restoration in spelt appears to be solely controlled by *Rf3*.

The order of SSR and SNP markers in the population CMS-Sperber/Primepi//Sperber is in agreement with the consensus map of Maccaferri et al. (2015), with the exception that *Xgwm264* was mapped more distal in the present study. Meaningful validation work in independent mapping populations and a diversity panel suggested that the marker *CAPS_IWB72107* and its underlying SNP *IWB72107* are suitable for the prediction of *Rf3* genotypes in marker-assisted selection. The discrepancy between phenotype and SNP genotype observed for Bauländer Spelz and Grey might be caused by recombination between *IWB72107* and *Rf3*, or by genes suppressing fertility restoration in these lines. The marker *CAPS_IWB72107* also produced null alleles, which are often associated with the presence of alien chromatin in host genomes (Brown-Guedira et al. 2003; Landjeva et al. 2006). The null alleles in the lines R1 and R3 might be due to introgressions of *T. timopheevii* (Bahl and Maan 1973), whereas the T1BL.1RS wheat-rye translocation is most likely responsible for the null alleles in Samir and Sirino (Zeller, personal communication). In addition, the two spelt cultivars also had null alleles for *Xpsp3000* and *Xgwm18*, two other markers located on chromosome 1BS supporting the presence of alien chromatin rather than a normal chromosome 1BS (data not shown). Since the maintainer lines 444-74, 539-74 and 563-76 were derived from wheat-rye translocation lines (Keydel, personal communication), their associated null

alleles are probably also due to the T1BL.1RS translocation.

In an initial screening for fertility restoration (data not shown), we observed that about 14% of European common wheat cultivars were able to restore the fertility of lines with *T. timopheevii* cytoplasm. However, the genes responsible for restoration in these lines remain unknown. In the present study, we used the SNP *IWB72107* to survey the frequency of *Rf3* genotypes in German breeding material and found that 8.8% of the analysed lines probably contained the fertility-restoring allele for *Rf3*. This marker frequency is similar to the findings of Zanke et al. (2014) who observed a frequency of 12% for the respective SNP allele in a panel of European wheat cultivars (Korzun, personal communication). Hence, we propose that *Rf3* might explain fertility restoration in a large proportion of European wheat cultivars. The observed allele frequency also indicated that the fertility-restoring *Rf3* allele probably has no positive effect on fitness and agronomic performance in common wheat. The SNP markers *IWB14060* and *IWB73447* linked to *Rf3* in the population CMS-Sperber/Primepi//Sperber indicated that the Primepi allele was present in 59.0 and 57.3% of the 524 German wheat breeding lines, respectively. Comparing these results to the allele frequency of *IWB72107*, we concluded that *IWB14060* and *IWB73447* are not diagnostic, since they would overestimate the presence of the fertility-restoring *Rf3* allele. In European spelt, we found that 66.7% of the analysed cultivars carried the fertility-restoring allele at the *Rf3* locus. The assumed *Rf3* genotypes of the spelt cultivars did not depend on their relationship to common wheat. This observation may support the hypothesis that neither the restoring nor the non-restoring alleles were recently introduced to spelt by crosses with common wheat. Since we found restoring and non-restoring *Rf3* genotypes among the two oldest cultivars Altgold and Oberkulmer Rotkorn, respectively, we concluded that both types of *Rf3* alleles have existed in European spelts for at least the last six decades. Whether the *Rf3* locus is limited to these two alleles and if there are species-specific alleles for common wheat and spelt remains unknown.

Little is also known about a possible relationship between *Rf3* and other restorer genes on the homoeologous group 1 chromosomes. Zhang et al. (2003) located a fertility-restoring gene on chromosome 1AS. It is possible that this gene is a homoeolog of *Rf3*. Moreover, Tsunewaki (2015) and Hohn and Lukaszewski (2016) reported a gene designated Rf^{multi} on chromosome 1BS, that restored male fertility of sterile lines with the cytoplasms of *Aegilops kotschyi* Boiss., *Aegilops mutica* Boiss. and *Aegilops uniaristata* Vis. Further research is required to investigate a possible relationship between the restorer gene on chromosome 1A, Rf^{multi} and *Rf3*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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