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RESEARCH PAPER

Identification of genes related to germination in aged maize seed by screening natural variability

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Abstract

Ageing reduces vigour and viability in maize inbred lines due to non-heritable degenerative changes. Besides non-heritable genetic changes due to chromosome aberrations and damage in the DNA sequence, heritable changes during maize conservation have been reported. Genetic variability among aged seeds of inbred lines could be used for association studies with seed germination. The objective of this study was to identify genes related to germination in aged seeds. The sweet corn inbred line P39 and the field corn inbred line EP44 were used as plant material. Bulks of living and dead seeds after 20 and 22 years of storage were compared by using simple sequence repeats (SSRs) and, when the bulks differed for a marker, the individual grains were genotyped. Differences between dead and living seeds could be explained by residual variability, spontaneous mutation, or ageing. Variability was larger for chromosome 7 than for other chromosomes, and for distal than for proximal markers, suggesting some relationships between position in the genome and viability in aged seed. Polymorphic SSRs between living and dead seeds were found in six known genes, including *pathogenesis-related protein 2*, *superoxide dismutase 4*, *catalase 3*, *opaque endosperm 2*, and *metallothionein1* that were related to germination, along with *golden plant 2*. In addition, five novel candidate genes have been identified; three of them could be involved in resistance to diseases, one in detoxification of electrophillic compounds, and another in transcription regulation. Therefore, genetic variability among aged seeds of inbreds was useful for preliminary association analysis to identify candidate genes.

Key words: Ageing, genetic variability, germination, *Zea mays*.

Introduction

During storage, ageing causes death of a variable number of seeds among maize (*Zea mays* L.) inbred lines, while surviving seeds of certain genotypes germinate and produce renewed seed with enhanced viability and vigour compared with the average of the inbred (Revilla *et al.*, 2006). From those results, it was concluded that there was heritable genetic variability for longevity within maize inbred lines, which allowed natural selection for viability and vigour during storage. Peto (1933), Fleming *et al.* (1964), Russell and Vega (1973), Bogenschutz and Russell (1986), and Chwedorzewska *et al.* (2002) also found heritable changes during the conservation of maize and rye (*Secale cereale* L.). Whittle (2006) stated that, on average, more heritable mutations occur per unit time during seed ageing than during the lifetime of the plant. In addition, non-heritable genetic changes due to chromosome aberrations and damage to DNA could also be associated with loss of germination in seeds during seed storage (Murata, 1991; Whittle, 2006).

Previous works normally relied on surviving seed when looking for induced variability; however, part of the variability hypothetically caused by ageing results in seed death. Therefore, dead seed provides valuable information on the effects of ageing. In this work allele frequencies were compared between dead and living seeds after long-term storage in order to determine genetic variability associated with germination and potential candidate genes. The objective of this study was to identify candidate genes associated with germination in aged maize seeds.

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Materials and methods

A 22-year-old seed lot of P39 obtained in 1983 (P39-83) by self-pollinating 19 plants was chosen as the plant material because this inbred line constitutes a unique material for studying variability within inbred lines (Tracy, 2000; Revilla *et al.*, 2005) and has relatively high longevity under cold storage conditions (4 °C and 50% relative humidity) (Revilla *et al.*, 2006). The sweet corn inbred line P39 was released by Smith in 1933 from Golden Bantam (an important sweet corn population). Considering that the analysis of one unique seed lot could not be generalized, another seed lot of P39 and a seed lot of the field corn line EP44 were used. Both lots were produced in 1985, P39-85 was obtained by self-pollinating 31 plants and EP44-85 by self-pollinating 19 plants. EP44 was released by Ordás in 1982 from Hembrilla×Queixalet (a singular Spanish landrace) and also showed longevity. The original percentage germination of these materials was 84% for P39-83, 85% for P39-85, and 95% for EP44-85.

The seed of P39-83 was screened with 226 primers distributed through the genome. From those simple sequence repeats (SSRs), a sample of 105 primers was used for characterization of P39-85 and EP44 seed lots. The SSR primers were chosen from http://www.maizegdb.org based on their distribution along the genome in the consensus map provided therein.

From each sample, P39-83, P39-85, and EP44-85, 300 seeds were placed on humid filter paper in Petri dishes (25 seeds per dish) at 25 °C for 7 d in the dark. Germination was 67% for EP44-85, 76% for P39-83, and 85% for P39-85. After germination, a sample of up to 96 coleoptiles from the germinated seeds and a sample of up to 96 embryos from the dead seeds were used for DNA extractions and subsequent analyses. When the sample of germinated or dead seeds was below 50, a further sample of seeds was germinated.

DNA was extracted from germinated plants from each sample, according to Liu and Whittier (1994) with modifications. DNA extractions from embryos were made using the Nucleospin® kit (BD Biosciences, Palo Alto, CA, USA). SSR amplifications were performed as described by Butrón *et al.* (2003). After amplification, SSR products were separated by electrophoresis using 1× TBE on a 6% nondenaturing acrylamide gel (~250 V for 3 h) (Shi *et al.*, 2001). Molecular markers (SSRs) associated with germination were identified in P39-83, P39-85, and EP44-85 by using bulk segregant analysis of living versus dead seeds (Quarrie *et al.*, 1999). When a marker was polymorphic between both bulks, all individuals from each bulk were genotyped for that marker.

SSR variability was classified into three main types: (i) different frequency of shared bands between dead versus living seeds; (ii) bands present exclusively in dead seeds; and (iii) bands present exclusively in living seeds. The homogeneity of band frequencies between dead and living seeds was checked by using a contingency table (Steel *et al.*, 1997). The possible bias factors from the expected random distribution of variability were checked by using χ^2 tests. The bias factors were (i) the position in the genome, specifically the distribution of SSR markers in the chromosomes and the position (distal versus proximal, distal being those SSRs located in the extreme bins of either chromosome arm, and proximal those located in the same bin as the centromere); and (ii) the repeat motif, specifically the type of repeat motif (simple versus composite), the number of nucleotides in the motif, and the nucleotide sequence of the motif (Table 1).

Results

Variability between living and dead seeds was observed for 3.8% of the SSR markers for P39-85, 8.5% for EP44-85, and 9.3% for P39-83 (Table 1). P39-83 showed significant type I variability between living and dead seeds for nine bands produced by six SSRs, significant type II variability for five bands of five SSRs, and type III variability for 12 SSRs. P39-85 showed significant type I variability between living and dead seeds for four bands produced by two SSRs, and type III variability for three SSRs. Finally, EP44 showed significant differences between living and dead seeds for the frequencies of six bands produced by five SSR primer pairs (type I variability), significant type II variability for the frequency of appearance of seven bands amplified by six SSR primer pairs, and type III variability for two SSRs (Table 2).

Amplifications of EP44 and P39-83 with primers *phi059* and *phi072* resulted in bands whose frequencies were different in living and dead seeds; bands were also different for each inbred line (Table 1). EP44 and P39-85 showed simultaneous, though unrelated variability for *umc1131*. Finally, the two inbreds and both origins of P39 showed variability simultaneously for *umc1169*.

The distribution of variability among chromosomes was not significantly heterogeneous, except for the seventh chromosome, because living and dead seeds differed for six of the 17 SSR primers located in chromosome 7 (Table 3). Concerning the position of the SSR marker in the chromosomes, there were 23 distal SSR markers across all chromosomes (located in the last bin of each arm) and six of them varied between living and dead seeds; while there were 22 proximal markers across all chromosomes (located in the centromere bin) and only one of them varied between living and dead seeds (Table 3).

The χ^2 for testing polymorphic SSRs was equally distributed between markers with simple and composite motifs, considering simple SSRs as those whose motif had a unique sequence of nucleotides repeated the same number of times (data not shown). Looking at SSRs with simple motifs, the number of nucleotides in the SSR motif did not cause any significant deviation in the distribution of variability, with the exception only of the unique six-nucleotide SSR, which was variable. The relative proportion of nucleotides in the repeat motif (proportion of adenine, thymine, A + T, or any other combination) did affect the

Table 1. SSR primers showing variability between dead and living naturally aged seeds of the maize inbred lines P39 and EP44, along with the position in the map, the repeated motif, the samples for which variation was detected, the possible amino acid that the triplet would codify if transcribed, and the gene or expressed sequence tag (EST) where the SSR is located, if any

SSR marker	Bin	Position[a]	Motif	Sample	Variability type[b]	Amino acid	Gene and ESTs
phi083	2.04	N	AGCT	P39-83	I and II		*pathogenesis-related protein 2*
phi072	4.01	N	AAAC	P39-83+EP44	I and II		*metallothionein 1*
phi076	4.11	D	AGCGGG	EP44	I and II	SerPro	*catalase 3*
phi112	7.01	N	AG	P39-85	I		*opaque endosperm 2*
phi069	7.05	N	GAC	EP44	I and II	Leu	
phi116	7.06	D	ACTG.ACG	EP44	I	Cys	
phi059	10.02	N	ACC	P39-83(+EP44)	I and II	Trp	
umc1169	1.04	N	TTA	All	I and II	Asn	*superoxide dismutase 4* + 2 ESTs
umc1147	1.07	N	CA	P39-85	III		2 ESTs
phi109642	2.04	N	ACGG	EP44	III		
umc1560	2.07	N	GC	EP44	II		1 EST
umc1394	3.01	N	AT	EP44	I and II		1 EST
umc1149	8.06	N	AG	P39-83	I		1 EST
umc1131	9.02	N	GCT	P39-85+EP44	III	Arg	2 ESTs
phi041	10.00	D	AGCC	P39-83	III		
bnlg1028	10.06	N	AG	P39-85	I and II		
umc1431	1.09	N	GCA	P39-83	III	Arg	2 ESTs
umc1737	1.11	N	AGA	P39-83	III	Ser	2 ESTs
umc1051	4.08	N	GA	P39-83	III		1 EST
umc1822	5.05	P	GA	P39-83	III		
umc1887	6.03	N	CGA	P39-83	III	Ala	
bnlg1702	6.05	N	AG	P39-83	III		
mmc0411	7.03	N	CT	P39-83	III		
umc1301	7.03	P	GCA	P39-83	III	Arg	2 ESTs
umc1015	7.03	P	GA	P39-83	III		
umc1359	8.00	D	TC	P39-83	III		1 EST
umc1318	10.01	P	GTC	P39-83	I	Gln	1 EST
umc1432	10.02	P	CTAG	P39	III		1 EST
umc1106	1.00	D	GAGA	P39	I		2 ESTs
umc1144	1.04	N	CT	P39	I and II		1 EST
umc2101	3.00	D	AG	P39	I and II		*golden plant 2*

[a] Position in the chromosome: D for SSRs located in the distal bins of either chromosome arm, and P for those located in the same bin as the centromere, otherwise N.
[b] Type of variation detected: I different frequency of shared bands between dead versus living seeds, II bands present exclusively in dead seeds, and III bands present exclusively in living seeds

distribution of variability. None of the sequence frequencies showed deviations from homogeneity for the two- or five-nucleotide motifs. Concerning the three-nucleotide SSRs, deviations from expectations were significant for the frequencies of the sequences AGA, GCA, and TTA. For four-nucleotide motifs, deviations were significant for ACGG, AGCT, CTAG, and GAGA.

Living versus dead 22-year-old seed of P39-83 differed for 21 SSRs, although for 12 SSRs differences were due to the lack of amplification among dead seeds. All remaining SSRs, except one, were either located in transcribed sequences [expressed sequence tags (ESTs)] with unknown function or in known genes, namely *pathogenesis-related protein 2* (involved in response to *Fusarium* seedling blight), *metallothionein 1* (response to stress due to heavy metals), *superoxide dismutase 4* (antioxidant enzyme), and *golden plant 2* (development of photosynthesizing maize tissues) (Table 1). Living versus dead seeds of P39-85 differed for six SSRs of type I and II, including those located in the genes *opaque endosperm 2* (involved in protein synthesis) and, again, *superoxide dismutase4* (antioxidant enzyme) and *golden plant 2*, and two located in ESTs. Finally, living versus dead seeds of EP44-85 differed for eight type I and II SSRs, of which three were located in ESTs, one in the gene *metallothionein 1* (response to stress by heavy metals), and two in genes involved in antioxidant enzyme synthesis, *catalase 3* and *superoxide dismutase 4*. It is noticeable that *superoxide dismutase4* variation was common to both inbreds and both origins of EP39. Both P39 and EP44 had variation for the gene *metallothionein 1* and for the markers *phi059* and *umc1131*. P39-83 and P39-85 had common variability for the markers *umc1169* and *umc2101*, located in the genes *superoxide dismutase 4* and *golden plant 2*, respectively, and for *umc1106* and *umc1144*.

Table 2. Variability (frequency of band for each type of variation and seed lot) between dead and living naturally aged seed of the maize inbred lines P39 and EP44

SSR marker	Band	P39-83					P39-85					EP44				
		Type I[a]		Type II		Type III	Type I		Type II		Type III	Type I		Type II		Type III
		L[b]	D	L	D		L	D	L	D		L	D	L	D	
phi083	1	1	0.97*	0	0.74*											
phi072	1			0	0.44*							0.54	0.3*	0	0.33*	
	2											0.59	0.67			
phi076	1											1	0.97*	0	0.22*	
	2													0	0.01	
phi112	1						1	0.83*								
	2						0.16	0.8*								
phi069	1											1	0.3*	0	0.06*	
phi116	1											0.01	0.25*			
phi059	1			0	0.13*									0	0.02	
	2			0	0.01									0	0.05*	
	3													0	0.07*	
umc1169	1	0.36	1*	0	0.04		0.59	0.98*				0.1	0.97*	0	0.03	
	2	1	0.5*				0.02	0.06				1	0.52*			
	3						1	0.62*								
umc1147	1										N[c]					
phi109642	1															N
umc1560	1													0	0.12*	
umc1394	1											1	0.99	0	0.19*	
umc1149	1	0.01	0.5*													
	2	1	0.96													
umc1131	1										N					N
phi041	1					N										
bnlg1028	1						1	0.96	0	0.04						
	2								0	0.04						
umc1431[d]	1					N										
umc1737[d]	1					N										
umc1051[d]	1					N										
umc1822[d]	1					N										
umc1887[d]	1					N										
bnlg1702[d]	1					N										
mmc0411[d]	1					N										
umc1301[d]	1					N										
umc1015[d]	1					N										
umc1359[d]	1					N										
umc1318[d]	1	0.28	0.73*													
	2	0.95	0.51*													
umc1432[d]	1					N										
umc1106[d]	1	0.09	1*													
	2	1	0.6*													
umc1144[d]	1	1	0.99	0	0.54*											
umc2101[d]	1	1	0.93*	0	0.73*											

[a] Type of variation: I different frequency of shared bands between dead versus. living seeds, II bands present exclusively in dead seeds, and III bands present exclusively in living seeds.
[b] L (=living) versus D (=dead) seeds.
[c] N, non-amplification in dead plants.
[d] These SSR primers were scored only in P39-83.
*Significant χ^2 at P=0.05.

The genomic DNA sequences for ESTs in which variable SSRs have been found (http://www.maizegdb.org) were BLASTed against the 'Filtered Gene Set' of the B73 sequence database (http://www.maizesequence.org/blast) and evidence gene sequences were identified (Table 4.)

Discussion

Variability between living and dead seed has been detected within inbred lines. Part of that variability (variability type III) could be attributed to chromosome aberrations or DNA damage during storage because bands present in the

Table 3. Deviations from the random distribution of polymorphic SSRs in the inbred lines P39 and EP44

Chromosome	Total no. of SSR markers	Variable SSR bands	
		Observed	Expected
Chromosome 1	27	6	3.7
Chromosome 2	23	3	3.2
Chromosome 3	16	2	2.2
Chromosome 4	28	3	3.8
Chromosome 5	22	1	3.0
Chromosome 6	25	2	3.4
Chromosome 7	17	6	2.3
Chromosome 8	23	2	3.2
Chromosome 9	18	1	2.5
Chromosome 10	27	5	3.7
	χ^2 Total = 16.9		
Position distal	23	6	3.6
Position proximal	22	1	3.4
	χ^2 Total = 3.4**		

χ^2 significant, at *P=0.05 and **P=0.01, respectively.

living individuals were not amplified among dead seeds; therefore, such variation does not provide reliable information. Variation for nine SSRs in EP39-83, six in EP39-85, and eight in EP44 did not seem to be related to inaccurate repair of DNA damage (variability type I and II). Type I variability should be due to the action of natural selection on residual genetic variability, while type II variability could be the consequence of new mutations occurring during seed storage. It cannot be certain that ageing is the cause of type II variability, but genetic changes are frequent during conservation of genotypes in germplasm banks (Fleming *et al.*, 1964; Russell and Vega, 1973; Bogenschutz and Russell, 1986; Murata, 1991; Revilla *et al.*, 2006). In order to establish a true cause–effect relationship, it is necessary to carry out experiments of controlled ageing and to check genetic changes in surviving and dead seeds.

The distribution of variability among chromosomes did not deviate from randomness except for chromosome 7, which showed a larger rate of variability than the other nine chromosomes (Table 3). Ordás *et al.* (2007) detected segregation distortion regions and reported previously published segregation distortion data in maize; one of the most persistent segregation distortions across experiments was in chromosome 7, precisely in bin 7.03, which is responsible for the bias on random distribution for chromosome 7 found in the present study. Apparently, there is a trend to show variability in bin 7.03.

Variability was significantly higher for distal markers than for proximal markers across all chromosomes (Table 3), which is in agreement with the theory that associates telomere stability and longevity (Monaghan and Haussmann, 2006). This result suggests that at least part of the variability could actually be explained by degradation of DNA due to ageing, although this experiment does not allow the definition of cause–effect relationships or a precise quantification of the amount of variability due to ageing or other causes.

The significant deviations from random distribution of variability between living and dead seeds related to number of nucleotides of the SSR motif or the sequence of the motif could have been found accidentally among so many comparisons. In contrast, Heckenberger *et al.* (2002) found more variability for di-repeat SSRs than for SSRs with longer repeat motifs in inbred lines. They also concluded that the mutation rate of SSRs depends on the repeat type, repeat number, and sequence (Heckenberger *et al.*, 2002).

Most of the SSRs varying between living and dead seeds did not correspond to triplets that could be translated to amino acids; actually only seven (one-third) of the variable SSRs in P39-83 had three-nucleotide motifs coinciding with amino acid triplets. Nevertheless, most of the SSRs differing between living and dead seeds are located in transcribed sequences (ESTs). These results contrast with the data reported by Kantety *et al.* (2002) showing that 1.5% of the ESTs in the collection of publicly available ESTs for maize contain SSRs. These results are not abnormal considering that variability between living and dead seeds was expected, which necessarily increases the chances of finding variability with some sort of expression in germination.

Residual or newly generated variability associated with viability of aged seed was found in several known genes which are directly or indirectly involved in germination (*pathogenesis-related protein 2*, *superoxide dismutase*, *catalase 3*, *opaque endosperm 2*, and *metallothionein 1*). The *pathogenesis-related protein 2* gene is involved in the response to *Fusarium* seedling blight, a disease that affects the rate of germination (Danielsen and Jensen, 1998). *Fusarium* could be involved in the germination ability of maize in the environment where these experiments were carried out because it is one of the main fungi infesting maize seeds in the northwest of Spain (Butrón *et al.*, 2006). Infestation by fungi is one of the main problems for germination of aged seed in our conditions, which, in turn, also relates to the gene *opaque endosperm 2* because Bass *et al.* (1992) have suggested that the coordination of *opaque 2*-controlled synthesis of a ribosome-inactivating protein (RIP) and the major seed storage proteins of the wild *opaque-2* allele provides nutritional benefits and protection against pathogen invasion.

Seed desiccation tolerance, measured as low loss of seed viability during storage, has been associated with an efficient operation of antioxidant systems such as superoxide dismutase (SOD) and catalase (CAT) because these enzymes act to prevent the accumulation of reactive oxygen species (ROS) during embryo development, desiccation, and early stages of germination (Bernal-Lugo *et al.*, 2000; Pukacka and Ratajczak, 2005; Cheng *et al.*, 2008; Huang *et al.*, 2009). ROS cause lipid peroxidation, protein denaturation, and DNA mutations (Mylona *et al.*, 2007). Finally, the metallothioneins are low molecular weight cysteine-rich proteins which bind metal in metal–thiolate clusters (Framond, 1991) and their possible role in germination has been proposed (Yuan *et al.*, 2008).

As most polymorphic genes between living and dead seeds were already known to be involved in germination, the procedure used in this study to look for candidate genes for

Table 4. Candidate genes for the ESTs in which SSRs showing variability between dead and living naturally aged seeds of the maize inbred lines P39 and EP44 were found

SSR marker	Variability type[a]	EST[b]	Candidate gene[c]	Location of candidate gene[c]	Likely function[c]
umc1147	III	p-std486020E10	GRMZM2G139222	AC202451.3:97319–100837	Includes a multicopper oxidase domain which participates in copper detoxification
umc1560	II	p-std687008E04	GRMZM2G179459	AC193317.3:54418–59747	Includes a variant SH3 and other domains found in disease resistance proteins
umc1394	I and II	p-std605090H09	GRMZM2G108459	AC217683:58455–66680	Disease resistance protein
umc1149	I	p-std486088C10	GRMZM2G098453	AC204676.3:69619–71628	Includes a GST domain; GST is involved in the detoxification of reactive electrophillic compounds by catalysing their conjugation to glutathione
umc1131	III	p-std605018G03	GRMZM2G154100	AC196056.3:92581–112981	Includes a zinc finger domain that could be involved in protein–protein interaction and be important in transcriptional activation–repression
umc1431	III	p-std606047B10	GRMZM2G004012	AC194892. 2:19694–20702	Includes a cupredoxin domain which participates in copper detoxification
umc1737	III	p-std660046G03	GRMZM2G082312	AC194381.3: 100837–103667	Unknown
umc1051	III	p-au02f03	GRMZM2G023333	AC212139.3: 126660–131155	Includes domains found in proteins which produce reactive oxygen species
umc1301	III	p-std603027C12	GRMZM2G108285	AC213618.3 :20468–23642	Includes a NUDIX hydrolase domain; NUDIX hydrolases have the ability to degrade potentially mutagenic, oxidized nucleotides while others control the levels of metabolic intermediates and signalling compounds
umc1359	III	PC0061754	GRMZM2G177733	AC211686.4:196718–197742	Unknown
umc1318	I	PC0094104	GRMZM2G093997	AC198290.3:18143–19575	Includes a domain present in plant lipid transfer, seed storage, and trypsin-α amylase inhibitor proteins
umc1432	III	p-std606046F03	GRMZM2G113073	AC198609.3:149578–154434	Includes domains related to RHO proteins involved in signal transduction
umc1106	I	p-std606021C12	No candidate gene		A low-confidence hypothetical gene which encodes an extensin protein was found. Extensins are involved in cell wall strengthening in response to mechanical stress
umc1144	I and II	p-std486039D01	GRMZM2G159069	AC194910.2:112244–114071	Includes a homeodomain-like domain; homeodomain proteins are transcription factors

[a] Type of variation: I different frequency of shared bands between dead versus living seeds, II bands present exclusively in dead seeds, and III bands present exclusively in living seeds.
[b] Name of the EST in which the SSR is located (http://maizegdb.org).
[c] Name, location, and likely function of the candidate gene obtained when the sequences of the ESTs were BLASTed against 'Filtered Gene Set' in B73 (http://www.maizesequence.org/blast).

germination using the genetic variability present in inbred aged seed seems promising. Therefore, the possible involvement in germination of genes such as *golden plant 2*, which acts as a transcriptional regulator of cellular differentiation in the maize leaf (Hall *et al.*, 1998), should be investigated, as should that of the unknown genes for which polymorphic markers have been found. As a first step, candidate genes for variable ESTs between dead and live seeds were identified. As expected, the likely functions of candidate genes associated with type III variability were diverse because that kind of variability could be a consequence of DNA damage. However, most genes presenting type I and II variability could encode enzymes acting to prevent abiotic stress and disease infections during germination.

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