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

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Received 27 February 2009; Revised 14 May 2009; Accepted 29 July 2009

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 Hembriš×Queixalet (a singular Spanish landrace) and also showed longevity. The original percentage germination of these materials was 84% for P39-85, 85% for P39-83, 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% non-denaturing 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 $\chi^2$ 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 $\chi^2$ 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
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 ACCG, 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 by 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 dismutase 4 (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 dismutase 4 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 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 | Positiona | Motif | Sample | Variability typeb | 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         | AGCGG | EP44    | I and II       | catalese 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         | ACTGACG | 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 |
| 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 |
| bnlgl1028  | 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 |
| umc1822    | 5.05| P         | GA    | P39-83  | III |
| umc1887    | 6.03| N         | GCA   | P39-83  | III | Ala |
| bnlgl1702  | 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.
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.)

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  | Type II | Type III | Type I  | Type II | Type III | Type I  | Type II | Type III |
|            |      | L       | D       | L       | D       | L       | D       | L       | D       | L       |
| phi083     | 1    | 1       | 0.97*   | 0       | 0.74*   | 0.54    | 0.3*    | 0       | 0.33*   |
| phi072     | 1    | 1       | 0.44*   | 0       |          | 0.59    | 0.67    |          |         |
| phi076     | 2    | 1       | 0.83*   | 0       |          | 1       | 0.97*   | 0       | 0.22*   |
| phi076     | 2    |          | 0.16    | 0.8*    |          | 0       | 0.01    |         |         |
| phi112     | 1    |          |         |         |          | 1       | 0.97*   | 0       | 0.06*   |
| phi069     | 1    |          |         |         |          | 0.01    | 0.25*   |         |         |
| phi116     | 1    |          |         |         |          | 0       | 0.02    |         |         |
| phi069     | 2    | 0       | 0.13*   | 0.54    | 0.3*    | 0.05*   |         |         |
| phi059     | 3    |          |         |         |          | 0       | 0.07*   |         |         |
| umc1169    | 1    | 0.36    | 1*      | 0       | 0.04    | 0.59    | 0.98*   | 0.1     | 0.97*   |
| umc1169    | 2    | 0       | 0.5*    | 0.02    | 0.06    | 1       | 0.52*   |         |         |
| umc1169    | 3    |          |         |         |          | 1       | 0.62*   |         |         |
| phi1147    | 1    |          |         |         |          |         | Nc      |         |         |
| phi109642  | 1    |          |         |         |          |         | N       |         |         |
| umc1560    | 1    |          |         |         |          |         | 0       | 0.12*   |         |
| umc1394    | 1    |          |         |         |          |         | 1       | 0.99    | 0       | 0.19*   |
| umc1149    | 1    | 0.01    | 0.5*    | 0       |          | 0.28    | 0.73*   | 0.95    | 0.51*   |
| umc1131    | 2    | 1       | 0.96    | 0       |          | 0.96    | 0.04    | 0       | 0.04    |
| phi041     | 1    |          |         |         |          |         | N       |         |         |
| bnlg1028   | 2    |          |         |         |          |         | 0       | 0.04    |         |
| umc1431b   | 1    |          |         |         |          |         |         | N       |         |
| umc1737b   | 1    |          |         |         |          |         | N       |         |         |
| umc1051b   | 1    |          |         |         |          |         | N       |         |         |
| umc1822b   | 1    |          |         |         |          |         | N       |         |         |
| umc1887b   | 1    |          |         |         |          |         | N       |         |         |
| bnlg1702b  | 2    |          |         |         |          |         | N       |         |         |
| mmc0411b   | 1    |          |         |         |          |         | N       |         |         |
| umc1301b   | 1    |          |         |         |          |         | N       |         |         |
| umc1015b   | 1    |          |         |         |          |         | N       |         |         |
| umc1359b   | 1    |          |         |         |          |         | N       |         |         |
| umc1318b   | 1    | 0.28    | 0.73*   | 0       |          | 0.28    | 0.73*   | 0.95    | 0.51*   |
| umc1432b   | 2    |          |         |         |          |         | 0.95    | 0.51*   |         |
| umc1106b   | 1    | 0.09    | 1*      | 0       |          | 0.09    | 1*      | 0.95    | 0.51*   |
| umc1144b   | 2    | 0       | 0.6*    | 0.09    | 0.54*   | 0       | 0.6*    | 0.95    | 0.51*   |
| umc2101b   | 1    | 0.93*   | 0.73*   | 0       |          | 0.93*   | 0.73*   | 0.95    | 0.51*   |

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.

L (=living) versus D (=dead) seeds.

N, non-amplification in dead plants.

These SSR primers were scored only in P39-83.

*Significant \( \chi^2 \) at \( P=0.05 \).

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
SSRs in the inbred lines P39 and EP44

during conservation of genotypes in germplasm banks
cause of type II variability, but genetic changes are frequent
during seed storage. It cannot be certain that ageing is
the consequence of new mutations occurring
on residual genetic variability, while type II variability
variability should be due to the action of natural selection
repair of DNA damage (variability type I and II). Type I
variation for nine SSRs in EP39-83, six in EP39-85,
therefore, such variation does not provide reliable informa-
living individuals were not amplified among dead seeds;
therefore, such variation does not provide reliable informa-
tion. 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). Orda’s 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 chro-
omosome 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 definit-
tion of cause–effect relationships or a precise quantification
of the amount of variability due to ageing or other causes.

### 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    |
| Position distal | 23     | 6       | 3.6    |
| Position proximal | 22    | 1       | 3.4    |

$\chi^2$ Total $= 16.9$

$\chi^2$ Total $= 3.4**$

$\chi^2$ significant, at *$P=0.05$ and **$P=0.01$, respectively.

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 pathogen-
esis-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 (Butron 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 nutri-
tional 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 superox-
ide 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 de-
naturation, 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 germina-
tion 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
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.

Acknowledgements

This research was supported by the Committee for Science and Technology of Spain (Project Cod. AGL2004-06776

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 typea | ESTb | Candidate gene c | Location of candidate gene | Likely functionc |
|------------|------------------|------|------------------|---------------------------|-----------------|
| umc1147    | III              | p-std486020E10 | GRMZM2G139222 | AC202451.3:97319–100837 | Includes a multicopper oxidase domain which participates in copper detoxification |
| umc1560    | II               | p-std87008E04 | 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 electrophilic compounds by catalysing their conjugation to glutathione |
| umc1131    | III              | p-std605018G03 | GRMZM2G154100 | AC196056:3.92851–112981 | 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-std605018G03 | GRMZM2G108459 | 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     | GRMZM2G103997 | AC198290:3:15143–15875 | 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 extin 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).
and AGL2007-64218) and Excma. Diputación Provincial de Pontevedra, Spain. VMR acknowledges a fellowship from the Spanish Ministry of Education and Science.

References

Bass HW, Webster C, O'Brian GR, Roberts JKM, Boston RS. 1992. A maize ribosome-inactivating protein is controlled by the transcriptional activator Opaque-2. The Plant Cell 4, 225–234.

Bernal-Lugo I, Camacho A, Carballo A. 2000. Effects of seed aging on the enzymatic antioxidant system of maize cultivars. In: Black M, Bradford KJ, Vázquez-Ramos J, eds. Seed biology: advances and applications. Wallingford, UK: CAB International, 151–160.

Bogenschutz TG, Russell WA. 1986. An evaluation for genetic variation within maize inbred lines maintained by sib-mating and self-pollination. Euphytica 35, 403–412.

Butrón A, Santiago R, Mansilla P, Pintos-Varela C, Ordás A, Malvar RA. 2006. Combining abilities of white maize (Zea mays L.) inbred lines for fumonisin contamination. Journal of Agricultural and Food Chemistry 54, 6113–6117.

Butrón A, Tarrio R, Revilla P, Malvar RA, Ordás A. 2003. Molecular evaluation of two methods for developing maize synthetic varieties. Molecular Breeding 12, 329–333.

Chwedorzewska KJ, Bednerek PT, Puchalski J. 2002. Studies on changes in specific rye genome regions due to seed aging and regeneration. Cellular and Molecular Biology Letters 7, 569–576.

Danielsen S, Jensen DF. 1998. Relationships between seed germination, fumonisin content, and Fusarium verticillioides infection in selected maize samples from different regions of Costa Rica. Plant Pathology 47, 609–614.

Fleming AA, Kozelnicky GM, Browne EB. 1964. Variation between stocks within long-time inbred lines of maize (Zea mays L.). Crop Science 4, 291–295.

Framond AJ. 1991. A metallothionein-like gene from maize (Zea mays). Cloning and characterization. FEBS Letters 290, 103–106.

Hall LN, Rossini L, Cribb L, Langdale JA. 1998. GOLDEN 2: a novel transcriptional regulator of cellular differentiation in the maize leaf. The Plant Cell 10, 925–936.

Heckenberger M, Bohn M, Ziegle JS, Joe LK, Hauser JD, Hutton M, Melchinger AE. 2002. Variation of DNA fingerprints among accessions within maize inbred lines and implications for identification of essentially derived varieties. I. Genetic and technical sources of variation in SSR data. Molecular Breeding 10, 181–191.

Huang H, Song S-Q, Wu X-J. 2000. Response of Chinese wampee axes and maize embryos to dehydration at different rates. Journal of Integrative Plant Biology 51, 67–74.

Kantety RV, La Rota M, Matthews DE, Sorrells M. 2002. Data mining for simple sequence repeats in expressed sequence tags from barley, maize, rice, sorghum and wheat. Plant Molecular Biology 48, 501–510.

Liu YG, Whittier RF. 1994. Rapid preparation of megabase plant DNA from nuclei in agarose plugs and microbeads. Nucleic Acid Research 22, 2168–2169.

Monaghan P, Haussmann MF. 2006. Do telomere dynamics link lifestyle and lifespan? Trends in Ecology and Evolution 21, 47–53.

Murata M. 1991. Cytogenetic changes during seed storage. In: Gupta PK, Tsuchiyat T, eds. Chromosome engineering in plants: genetic, breeding, evolution. Part A. Amsterdam, The Netherlands: Elsevier Science Publishers, 211–228.

Mylona PV, Poldoros AN, Scandalios JG. 2007. Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella in maize. Journal of Experimental Botany 58, 1301–1312.

Ordas B, Romay MC, Hill WG. 2007. Effect of selection on the heterozygosity of inbred lines of maize. Molecular Breeding 20, 117–129.

Peto FH. 1933. The effect of aging and heat on the chromosomal mutation rates in maize and barley. Canadian Journal of Research 9, 261–264.

Pukacka S, Ratajczak E. 2005. Production and scavenging of reactive oxygen species in Fagus sylvatica seeds during storage at varied temperature and humidity. Journal of Plant Physiology 162, 873–885.

Quarrie SA, Lazic-Jancic V, Kovacevic D, Steed A, Pekic S. 1999. Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. Journal of Experimental Botany 50, 1299–1306.

Revilla P, Abuín MC, Malvar RA, Soengas P, Ordás B, Ordás A. 2005. Genetic variation between Spanish and American versions of sweet corn inbred lines. Plant Breeding 124, 268–271.

Revilla P, Velasco P, Malvar RA, Cartea ME, Ordás B, Ordás A. 2006. Variability among maize (Zea mays L.) inbred lines for seed longevity. Genetic Resources and Crop Evolution 53, 771–777.

Russell WA, Vega OU. 1973. Genetic stability of quantitative characters in successive generations in maize inbred lines. Euphytica 22, 172–180.

Shi J, Ward R, Wand D. 2001. Application of a high throughput, low cost, nonadenaturing polyacrylamide gel system for wheat microsatellite mapping. National Fusarium Blight Forum, USA.

Steel RDG, Torrie JH, Dickey DA. 1997. Principles and procedures in statistics: a biometrical approach, 3rd edn. New York: McCraw Hill.

Tracy WF, Talbert L-E, Gerdes JT. 2000. Molecular variation and F1 performance among strains of the sweet corn inbred P39. Crop Science 40, 1763–1768.

Whittle CA. 2006. The influence of environmental factors, the pollen: ovule ratio and seed bank persistence on molecular evolutionary rates in plants. Journal of Evolutionary Biology 18, 302–308.

Yuan J, Chen D, Ren YJ, Zhang XL, Zhao J. 2008. Characteristic and expression analysis of metallothionein gene, OsMT2b, down-regulated by cytokinin suggest functions in root development and seed embryo germination of rice. Plant Physiology 146, 1637–1650.