Journal of Experimental Botany
doi:10.1093/jxb/erw018

RESEARCH PAPER

An apomixis-linked *ORC3*-like pseudogene is associated with silencing of its functional homolog in apomictic *Paspalum simplex*

Lorena A. Siena[1,2], Juan Pablo A. Ortiz[1,2], Ornella Calderini[3], Francesco Paolocci[3], Maria E. Cáceres[3], Pankaj Kaushal[3,*], Simone Grisan[3], Silvina C. Pessino[1,2] and Fulvio Pupilli[3,†]

[1] Laboratorio de Biología Molecular, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, (S2125ZAA) Zavalla, Argentina
[2] Instituto de Investigaciones en Ciencias Agrarias de Rosario (IICAR-CONICET-UNR), Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, (S2125ZAA) Zavalla, Argentina
[3] Istituto di Bioscienze e Biorisorse (IBBR-CNR), via della Madonna alta 130, I-06128 Perugia, Italy

* Present address: Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi 284003, India.
† To whom correspondence should be addressed. E-mail: fulvio.pupilli@ibbr.cnr.it

Received 6 November 2015; Revised 5 January 2016; Accepted 8 January 2016

Editor: Daphne Goring, University of Toronto

Abstract

Apomixis in plants consists of asexual reproduction by seeds. Here we characterized at structural and functional levels an apomixis-linked sequence of *Paspalum simplex* homologous to subunit 3 of the ORIGIN RECOGNITION COMPLEX (ORC3). ORC is a multiprotein complex which controls DNA replication and cell differentiation in eukaryotes. Three *PsORC3* copies were identified, each one characterized by a specific expression profile. Of these, *PsORC3a*, specific for apomictic genotypes, is a pseudogene that was poorly and constitutively expressed in all developmental stages of apomictic flowers, whereas *PsORC3b,* the putative functional gene in sexual flowers, showed a precise time-related regulation. Sense transcripts of *PsORC3* were expressed in the female cell lineage of both apomictic and sexual reproductive phenotypes, and in aposporous initials. Although strong expression was detected in sexual early endosperm, no expression was present in the apomictic endosperm. Antisense *PsORC3* transcripts were revealed exclusively in apomictic germ cell lineages. Defective *orc3* mutants of rice and Arabidopsis showed normal female gametophytes although the embryo and endosperm were arrested at early phases of development. We hypothesize that *PsORC3a* is associated with the down-regulation of its functional homolog and with the development of apomictic endosperm which deviates from the canonical 2(maternal):1(paternal) genome ratio.

Key words: Apomixis, gene silencing, origin recognition complex, *Paspalum*, seed development.

Introduction

The expression 'gametophytic apomixis in angiosperms' refers to the formation of a female gametophyte (i.e. the embryo sac) from an unreduced cell and the subsequent development of a clonal embryo from a 2*n* egg by parthenogenesis (Nogler, 1984). Whether the unreduced embryo sac arises from a generative cell or from a somatic cell is a very important distinction. In diplospory, the unreduced embryo sac originates from the megaspore mother cell, either directly by mitosis or

indirectly by a modified meiosis, whereas in apospory, unreduced embryo sacs develop from somatic cells of the ovule, usually belonging to the nucellus (Ozias-Akins and van Dijk, 2007). From a plant breeding perspective, apomixis could allow the fixation of heterotic vigor in hybrid seeds (Hanna, 1995).

Although apomixis is widespread in the plant kingdom, it is present in just a few crops. Several attempts to transfer this trait into major crops from wild relatives have repeatedly failed, giving rise to partially fertile, agronomically unsuitable lines (Vielle-Calzada *et al.*, 1996). Plants producing clonal progeny by seeds have been obtained by engineering an artificial apomixis system in Arabidopsis (Marimuthu *et al.*, 2011), representing a first proof-of-principle of the possibility to induce apomixis in a sexual background. However, since these mutants still rely on crossing to express the trait, they cannot be considered as genuine apomictic plants (Pupilli and Barcaccia, 2012). Furthermore, despite several candidate genes for apomixis having been described in natural apomictic systems (Corral *et al.*, 2013; Mau *et al.*, 2013; Hand and Koltunow, 2014), only one of them was able to trigger parthenogenesis in a sexual background (Conner *et al.*, 2015). Consequently, as apomixis as a whole is probably controlled by several genes, there is still a need to obtain insights into the genetic mechanisms underlying asexual reproduction in natural apomicts to verify whether candidate genes isolated from these species could serve for developing a stable and universal apomixis system.

The genus *Paspalum* presents a number of characteristics that make it unique to study apomixis (Ortiz *et al.*, 2013) including the availability of an efficient plant transformation system (Mancini *et al.*, 2014). Several candidate genes for apomixis were identified in both *P. simplex* and *P. notatum* using different strategies (Pessino *et al.*, 2001; Calderini *et al.*, 2006; Laspina *et al.*, 2008; Polegri *et al.*, 2010), and functional studies of the most interesting genes are ongoing (Felitti *et al.*, 2011; Podio *et al.*, 2014*b*; Siena *et al.*, 2014). However, according to our view, a gene associated with apomixis should fit the following criteria in order to be considered a promising candidate as a (one of the) genetic determinant(s) of the trait: (i) homology with genes related to reproductive development; (ii) co-segregation with apomixis; (iii) differential expression between apomictic and sexual flowers; and (iv) availability of mutants for its homolog in sexual model species showing altered reproductive phenotypes.

One of the genes present on an apomixis-linked bacterial artificial chromosome (BAC) clone of *P. simplex* showed high similarity to subunit 3 of the *ORIGIN RECOGNITION COMPLEX* (*ORC3*) (Bell and Dutta, 2002). ORC is a six subunit protein complex that, together with other factors, constitutes the pre-replicative complex (pre-RC) which licences DNA to replicate. In addition to DNA replication, ORC proteins have a role in chromatin structure modification and gene silencing in higher eukaryotes, fungi, and metazoans (DePamphilis, 2003; Remus and Diffley, 2009). In Arabidopsis, one of the ORC components, ORC2, interacts with the Polycomb group protein MEDEA that has a role in arresting the central cell proliferation prior to fertilization of polar nuclei (Collinge *et al.*, 2004). MEDEA-defective mutants show spontaneous endosperm development, a characteristic of autonomous apomixis (Grossniklaus *et al.*, 1998).

Here, we report on the structure, comparative mapping, expression, and functional analyses of *PsORC3* in apomictic and sexual *Paspalum* species and postulate a role for this gene in the development of apomictic seeds in *P. simplex.*

Materials and methods

Plant material

The following subpopulations belonging to four different *Paspalum* species, all formed by tetraploid individuals ($2n=4x=40$) and segregating for apomixis, were used: (i) *P. simplex*, 34 apomictic and 53 sexual BC_1 plants (Pupilli *et al.*, 2001); (ii) *P. notatum*, 10 apomictic and 10 sexual F_1 plants (Martínez *et al.*, 2001); (iii) *P. malacophyllum*, six apomictic and 19 sexual F_1 plants (Pupilli *et al.*, 2004); and (iv) *P. procurrens*, four apomictic and 30 sexual F_1 plants (Hojsgaard *et al.*, 2011). Segregating seeds of the rice insertional mutant lines Ne9014, Ng1015, and RdSpm2126B, carrying insertions in the *ORC3* gene (LOC_Os10g26280.1) were obtained from the Rice Genome Resource Centre of the National Institute of Agrobiological Sciences, Japan (Ne9014 and Ng1015; Miyao *et al.*, 2003) and the Department of Plant Biology, University of California, Davis CA, USA (RdSpm2126B; Kumar *et al.*, 2005). The Arabidopsis T-DNA mutant line GABI_270G02, defective for the same gene (At5G16690), was obtained from the GABI-Kat collection at the Nottingham Arabidopsis Stock Centre (Kleinboelting *et al.*, 2012). Rice mutant lines were grown as suggested (https://tos.nias.affrc.go.jp/doc/condition.html.en). *Paspalum* plants were maintained in a greenhouse under routine breeding practices, whereas Arabidopsis plants were grown in a growth chamber (22 °C for 16 h daylight and 18 °C for 8 h in the dark).

Southern blots, BAC partial sequencing, and cloning of genomic PsORC3

*Eco*RI or *Xba*I restriction enzymes (New England Biolabs; www.neb.com/) were used for Southern blot analysis in *Paspalum* species and in insertional mutant lines of rice, respectively, as reported (Pupilli *et al.*, 2001). The apomixis-linked BAC 366H1, isolated from a genomic library of apomictic *P. simplex* (Calderini *et al.*, 2011), was used for identifying candidate genes for apomixis. A *Hin*dIII subclone library of BAC 366H1 was cloned in the pBluescript vector and amplified using DH10B electrocompetent cells (www.lifetechnologies.com/) through standard procedures (Sambrook *et al.*, 1988). Inserts were sequenced with T7 and T3 primers using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; www.lifetechnologies.com/). The reactions were then run on an ABI PRISM® 3130 Genetic Analyzer. Based on sequence data, the GenomeWalker™ (GW) procedure was used on genomic DNA of apomictic and sexual plants to isolate the apomixis- and sex-specific genes using either 3'- or 5'-oriented primer pairs (see Supplementary Table S1 at *JXB* online) according to the manufacturer's instruction manual (Clontech; http://www.clontech.com/).

RNA extraction, reverse transcription–PCR, and cloning of P. simplex ORC3 cDNAs

RNA from sexual and apomictic flowers of *P. simplex* and from mutant and wild-type (WT) flowers of rice was isolated at the anthesis stage using a Nucleo Spin RNA Plant Isolation kit (Macherey-Nagel; http://www.mn-net.com/). A DNase

I (Ambion; www.lifetechnologies.com/) treatment was carried out to remove traces of DNA contamination from RNA, according to the manufacturer's instruction. The absence of contaminant DNA was tested by PCR amplification of DNase I-treated RNAs in the presence of the universal rDNA primer pair ITS1/ITS4 as described (Polegri *et al.*, 2010). RNA was retrotranscribed with SuperScript® III Reverse Transcriptase (Life Technologies) according to the manufacturer's protocol using an oligo$(dT)_{18}$ primer. The resulting cDNAs were used to amplify the complete coding sequence of the three *ORC3* genes. cDNA from apomictic plants was used as the template to amplify *PsORC3a* using the primer pair OrApofor/OrAporev (Supplementary Table S1), and cDNA from sexual plants to amplify *PsORC3b* and *PsORC3c* using the primer pairs OrSexfor/OrSexrev (Supplementary Table S1). PCR conditions were: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 3 min; and a final extension at 72 °C for 7 min. Rice cDNAs were used to compare the transcript abundance of the rice homolog of *PsORC3* in mutant lines and WT flowers using the β-tubulin gene as the normalizer. Primer pairs used were Os1921f/OsOrc3stoprev for *ORC3* and OsTubFor/OsTubRev for β-tubulin (Supplementary Table S1). The PCR conditions were: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 55°C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. Amplicons were cloned in pGEM®-T Easy vectors (Promega; www.promega.com) and sequenced from internal primers (Supplementary Table S1) as reported above.

Sequence data deposition

The sequence data reported in this paper have been deposited in the EMBL-EBI database (http://www.ebi.ac.uk/ena) [accession nos (genomic, mRNA) LN832398, LN832399 for *PsORC3a*; LN832402, LN832403 for *PsORC3b*; and LN832400, LN832401 for *PsORC3c*].

Phylogenetic analysis

All amplified fragments from *P. simplex* were queried against the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database using the BLAST 2.2.25 tool (Altschul *et al.*, 1997) to identify similar sequences. BLASTn and BLASTx were used to compare nucleotides and translated sequences, respectively. The corresponding proteins and ORFs were deduced using the gene prediction softwares ExPASy (Gasteiger *et al.*, 2003) (http://web.expasy.org/translate/) and Pfam (Finn *et al.*, 2013) at the EMBL-EBI website (http://pfam.xfam.org/). Sequences showing the most significant nucleotide and/or amino acid sequence homology were used in multiple sequence alignments with ClustalW (Chenna *et al.*, 2003) (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The matrix obtained from multiple alignments of plant ORC proteins was used to perform a phylogenetic analysis using Mega6 software (Tamura *et al.*, 2013). Five thousand bootstrap replicates were performed to obtain an unrooted consensus tree.

Real-time RT–PCR

Staging was estimated by correlating morphological characteristics with cytoembryological development (Cáceres *et al.*, 2001). A denotes apomictic and S sexual ovules (Fig. 3). Of these, stage I (flower morphology/developmental stage) comprises inflorescences completely enveloped in the flag leaf sheath/no differentiated meiotic cells or first appearance of the megaspore mother cell (A and S); stage II, 1–2 d before anthesis as predicted by anthesis initiation in apical racemes/development of eight-nucleated (S) and aposporic *Hieracium*-type (A) embryo sacs; stage III, anthesis/egg and central cell fertilization; stage IV, 1–3 d after anthesis/fertilization accomplished, early stages of embryo and endosperm development; stage V, 5 d after anthesis/early stages of seed formation (A and S); and stage VI, 10 d after anthesis/seed completely developed (A and S). Two different genotypes for each developmental stage of both sexual and apomictic phenotypes were considered. Florets used for RNA extraction were left to open pollinate in the growth chamber so that they shared a common pool of pollen. Total RNA was extracted as described above from each developmental stage considered. cDNAs from apomictic and sexual flowers were synthesized from 4 µg of total RNA using SuperScript III H-Reverse Transcriptase (Invitrogen; www.lifetechnologies.com/) and 100 pmol of random hexamers (Pharmacia Biotech; www.gelifesciences.com/) following the manufacturer's protocol. Primers and TaqMan probes for real-time amplification of the housekeeping gene and sexual and apomict *PsORC3* targets were designed on single nucleotide polymorphisms (SNPs) and indels with the help of the OligoExpress Software (Applied Biosystems). The synthesis of primers and probes was performed by Applied Biosystems UK; their sequences are shown in Supplementary Table S1. A cytidine deaminase (*PsCDA*; GeneBank accession no. AM400871) gene was used as the internal housekeeping gene using the primers and amplification conditions reported by Polegri *et al.* (2010). The successful synthesis of cDNA was proved by the amplification of 1:10 (v/v) diluted cDNAs with primers PSCTfw and PSCTrev, designed on the ORF of the *PsCDA* gene. The allele specificity and amplification efficiency of each primer–probe combination were tested against a serial dilution of plasmids harboring the genes of interest and on genomic DNA of the whole BC_1 population of *P. simplex*. qRT–PCR was performed using the TaqMan® Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instruction in a total volume of 20 µl per reaction. For each developmental stage, two independent RNA isolations from two different genotypes were performed. Then, for each RNA sample, two reverse transcription reactions were performed and pooled. An aliquot of 4 µl of 1:10 diluted cDNA pools was used in the qRT–PCR. Reactions were then run and analyzed on the 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's instruction. Four technical replicates were run for each cDNA sample, and no-template controls were incorporated in all assays. For each reaction, the threshold cycle value (Ct) was determined by setting the threshold within the logarithmic amplification phase. The relative expression levels of *PsORC3* targets in each genotype at each developmental stage were then calculated after normalization to *PsCDA*, by using the ΔΔ-Ct method according to the User Bulletin 2, ABI Prism 7700 sequence detection systems (Livak and Schmittgen, 2001). For each given target gene, the transcript quantity of the apomixis-specific gene in apomictic flowers at stage I was used as a calibrator, and arbitrarily set to 1, and all other samples were expressed in relation to this control sample. For each stage and each phenotype, the reactions were repeated twice with two different cDNA preparations, and one of the experiments was plotted.

In situ hybridization

A *PsORC3* probe was amplified from 100 ng of genomic DNA from an apomictic genotype of *P. simplex* using the primer pair Orfor1 and Orrev1 (Supplementary Table S1), and the derived 614 bp amplicon was cloned into a pGEM-T easy vector (Promega). Spikelets of sexual and apomictic *P. simplex* and *P. notatum* genotypes were collected at stages I, II, III, and IV and used for *in situ* hybridization as reported (Siena *et al.*, 2014).

Reverse genetics

Plantlets of lines Ne9014, Ng1015, and RdSpm2126B were screened by PCR, and amplicons were sequenced to validate the insertion sites. Primers are reported in Supplementary Table S1. Southern blot analyses were carried out for the lines Ne9014 and Ng1015 to distinguish between the heterozygous, homozygous, and WT state of the insertions according to the supplier's recommendations.

Transposon- and gene-specific primers used to develop radiolabeled probes are reported in Supplementary Table S1. Mutant and WT alleles of the RdSpm2126B line were amplified with appropriate primer pairs (dSpm3′fw/OsORC3dSpMrev and OsORC3dSpMfor/OsORC3dSpMrev respectively, Supplementary Table S1) designed as suggested (http://sundarlab.ucdavis.edu/rice/blast/blast.html). Arabidopsis mutant plantlets of the T-DNA insertion line GABI_270G02 were analyzed with the primer pair pAC161T-DNAPCR/GSP270G02 to confirm the insertion and with the pair GSP270G02/GSP270G02left, for amplifying the WT allele. The primer pAC161T-DNASEQ was used for sequencing the insertion (Supplementary Table S1). Paleas and lemmas of WT and homozygous mutant spikelets of rice were removed with fine scissors, and reproductive structures were observed under a stereomicroscope. Kruskal–Wallis one-way ANOVA was conducted on the aborted seed ratio in heterozygous selfed progeny in rice. Significant differences among WT, heterozygous, and homozygous plants were determined using multiple comparison Dunn's post-test. All statistical analyses were performed using the software Graphpad InStat® (Motulsky, 2003). Comparative histological analysis of developing seeds at anthesis and 10 d after anthesis was performed according to the safranine–fast green method (Cáceres *et al.*, 1999). Arabidopsis siliques at stage 6.50 (Boyes *et al.*, 2001) were opened under a stereomicroscope and ~1000 seeds were counted to evaluate the percentage abortion. To determine the phenotype of *orc3* mutant embryos, seeds were excised from siliques of heterozygous plants and cleared in Hoyer's solution (Liu and Meinke, 1998). The seeds were then mounted and observed for defects in embryogenesis with a Zeiss Axiophot microscope using differential interference contrast (DIC).

Results

Cloning, characterization, and mapping of ORC3 sequences and of their deduced proteins

To identify candidate genes for apomixis, a subclone library from the *P. simplex* BAC genomic clone 366H1, including a 112 kb long fragment belonging to the apomixis-controlling region (ACR; Calderini *et al.*, 2011), was produced by *Hin*dIII digestion. Of the 63 recombinant clones, one harbored a 3416 bp insert homologous to the *ORC3* gene. Consequently, it was named *PsORC3a*. Internal primers were designed (Supplementary Table S1) to clone the 3' and 5' ends of the gene by the GW procedure. The correct assembly of the resulting full-length gene, consisting of 2164 nucleotides, was validated by re-sequencing using end-to-end specific primers (Supplementary Table S1). To verify the presence of *PsORC3a*-like genes in sexual genotypes, the genomic DNA of a sexual BC_1 plant was amplified using primers designed within the ORF (Orfor2/Orrev2, Supplementary Table S1). The amplification reaction showed a fragment of the same size as that observed in the apomictic genotype. After sequence verification, the sexual amplicon was extended to the 3' and 5' ends by GW using appropriate primer pairs (Supplementary Table S1). When the resulting contig was cloned and re-sequenced, two different copies, *PsORC3b* and *PsORC3c* of 2184 and 2165 nucleotides, respectively, were identified. Sequence analysis of *PsORC3b* and *PsORC3c* revealed significant similarities (ID 91%; E-value 0.0) with *PsORC3a*. Alignments with ClustalW (Supplementary Fig. S1) showed that *PsORC3* sequences differed mainly at the beginning of the 5' region, where *PsORC3a* has two deletions of 14 and six nucleotides (nucleotides 54–68 and 129–135, respectively) compared with the consensus sequence. Of these, the former is absent in both *PsORC3b* and *PsORC3c* and the latter is missing in *PsORC3b* but is larger (12 nucleotides, between nucleotides 125 and 137 of the consensus sequence) in *PsORC3c.* To confirm the presence of different *ORC3* copies in the *P. simplex* genome, a Southern blot analysis was carried out on DNA from apomictic and sexual BC_1 plants using the Orfor2/Orrev2 amplicon as probe (Fig. 1). The restriction enzyme used (*Eco*RI) had no recognition sites in any of the three clones identified. As expected, three hybridizing fragments were evidenced, of which one, spanning ~4.5 kb, was specific to apomictic plants whereas the other two were common to both phenotypes. As the two common fragments were not polymorphic between the parental lines of the segregating population, no information on possible allelic relationships between them, or with the apomixis-specific one could be inferred. Therefore, we call these sequences 'copies' rather than alleles. To verify the presence of an apomixis-linked *ORC3* gene in other *Paspalum* species, the same probe was hybridized against the DNA from apomictic and sexual hybrids belonging to mapping populations of *P. procurrens*, *P. malacophyllum*, and *P. notatum*. The hybridizing patterns showed a band co-segregating in coupling with apomixis in all the three species tested (Supplementary Fig. S2).

To verify whether these genes were transcribed in reproductive organs, RT–PCR was performed on RNA isolated from flowers of one apomictic and one sexual BC_1 plant, using the same sexual- or apomixis-related end oligonucleotides used for genomic DNA amplification. We noticed that all three copies were expressed in flowers (not shown). Moreover, as the sequence of all the three cDNAs amplicons matched perfectly to that of their genomic counterparts, we concluded that the corresponding genes did not contain introns.

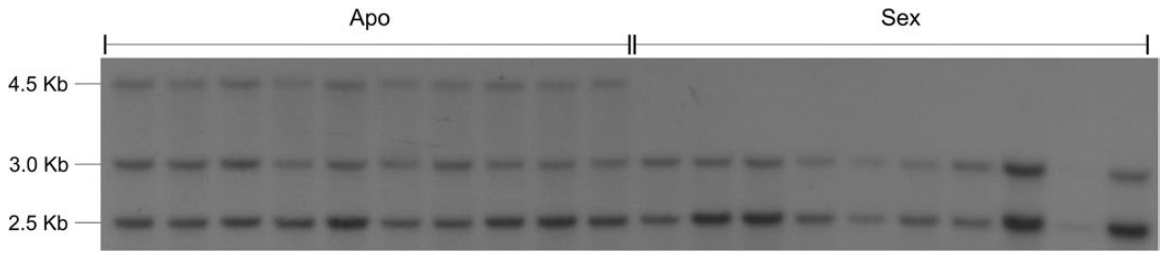

Fig. 1. Banding pattern of a *PsORC3* probe hybridized with apomictic (Apo) and sexual (Sex) *P. simplex* genomic DNA digested with *Eco*RI. As no *Eco*RI sites were present within the genomic sequence of *PsORC3*, the three RFLP (restriction fragment length polymorphism) fragments detected probably represent the three copies of the gene. *PsORCa* is included in the apomixis-linked fragment of 4.5 kb.

BLASTx analysis at the NCBI website using the *PsORC3a* sequence as query showed two significant alignments (ORF +1 between nucleotides 760 and 1053, E-value 0.0; and ORF +2 between nucleotides 170 and 775, E-value 0.0) with the eukaryotic ORC subunit 3 superfamily N-terminus (ORC3_N in Fig. 2). However, in both ORFs, the nucleotide sequence included several stop codons, suggesting that *PsORC3a* is a pseudogene, expressing an mRNA that is likely to be unable to be translated into a functional protein (Fig. 2A). A similar analysis with the *PsORC3b* sequence displayed high similarity with the complete domain of the ORC subunit 3 superfamily (ORF +1 between nucleotides 154 and 1077, E-value $4.00e^{-46}$; Fig. 2B). The predicted protein, starting from the initial methionine, contains 727 amino acids. Similar results were obtained with *PsORC3c*, although the expected protein contains only 589 amino acids due to a premature stop codon at nucleotide 1770 (Fig. 2C). The translated amino acid sequences from *PsORC3b* and *PsORC3c* were compared with 12 ORC3 proteins from several plant species to analyze the level of conservation of the different domains. The alignment of proteins showed that the regions 1–110 and 380–620 presented a low level of conservation, whereas the region 120–380 and the C-terminal fragment (736–771) displayed a middle and high level of conservation, respectively (Supplementary Fig. S3). Furthermore, similarity for PsORC3b and for the truncated PsORC3c started at consensus sequence amino acid 1 and finished at 727 and 589, respectively (Supplementary Fig. S3). A phylogenetic analysis was conducted by comparing the PsORC3 proteins with 15 ORC3 sequences from different organisms (Supplementary Fig. S4). As expected, PsORC3b and PsORC3c formed one cluster with ORC3 proteins from *Zea mays*, *Sorghum bicolor*, and *Setaria italica*, close to another cluster formed with other monocots (*Oryza sativa*, *Oryza brachiantha*, *Brachypodium distachyum*, and *Triticum urartu*). These sequences clearly differed from those of ORC3 proteins from the dicotyledonous species (*Glycine max*, *Arabidopsis thaliana*, *Ricinus communis*, *Theobroma cacao*, *Beta vulgaris*, and *Gossypium arboretum*) and from those of humans and yeasts.

Overall, it can be concluded that one homolog of the *ORC3* gene is linked to apomixis in several *Paspalum* species. In *P. simplex*, this gene exists as three different copies, of which *PsORC3a* is a pseudogene specific for apomicts expressing an RNA transcript unlikely to be translated in a functional protein, whereas *PsORC3b*, probably coding for a highly conserved functional protein, together with *PsORC3c*, coding for a truncated protein, are common to both apomictic and sexual plants.

PsORC3 expression analysis

In order to determine the expression patterns of *PsORC3* during flower development, copy-specific qRT–PCR assays were carried out at six different development stages. As a step prior to real-time PCR experiments, primer pairs and TaqMan probes were developed to generate copy-specific amplicons. These oligonucleotides were tested on the genomic DNAs of the whole *P. simplex* BC_1 mapping population (Pupilli *et al.*, 2001) to validate their specificity: *PsORC3a* was found to be specific to apomictic genotypes, *PsORC3b* was always present in both apomictic and sexual genotypes, and *PsORC3c* showed an extent of segregation which was independent from the reproductive mode (not shown). qRT–PCR analyses were then performed to delineate the transcriptional profiles of each of the three *PsORC3* copies at six developmental stages in both apomictic and sexual plants (Fig. 3). The non-coding

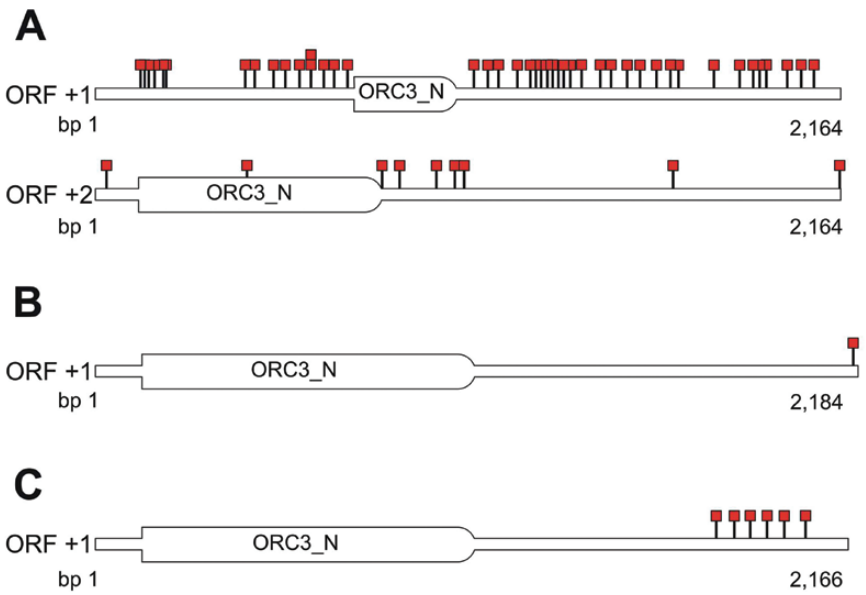

Fig. 2. Graphical representation of NCBI BLASTx significant alignment of three *PsORC3* genes to the eukaryotic ORC subunit 3 superfamily N-terminus. The two most significant alignments of *PsORC3a* (2164 nucleotides), ORF +1 (nucleotides 760–1053, E-value 0.0) and ORF +2 (nucleotides 170–755, E-value 0.0), show several stop codons (tags) predicting non-functional proteins (A). The most significant alignment of *PsORC3b* (2184 nucleotide), ORF +1 (nucleotides 154–1077, E-value $4.00e^{-46}$), predicts a 727 amino acid protein (B) whereas for *PsORC3c* (2166 nucleotides), the most significant prediction, ORF +1 (nucleotides 154–1077, E-value $4.00e^{-46}$), produced a 589 amino acid protein due to the presence of a premature stop codon at nucleotide 1770 (C). (This figure is available in colour at *JXB* online.)

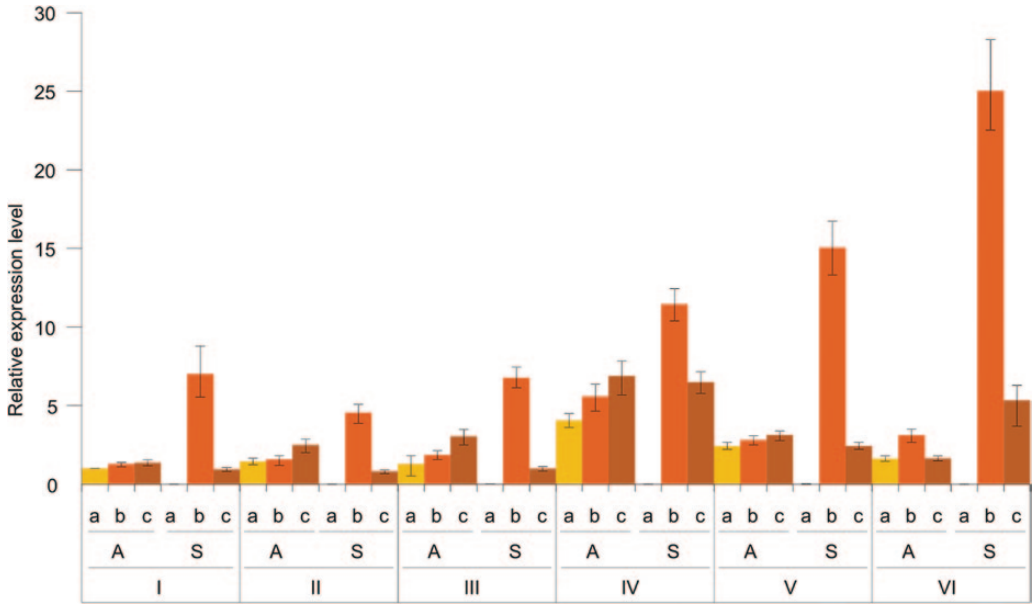

Fig. 3. Determination of the transcriptional profile of three *PsORC3* genes through qRT–PCR assay carried out at six different development stages (I–VI, see the Materials and methods for description of the stages) of sexual (S) and apomictic (A) flowers. a, b, and c refer to *PsORC3a*, *PsORC3b*, and *PsORC3c*, respectively, and error bars indicate the SEs. Each bar represents the mean of four replicates, and the relative expression level is referred to the expression value of *PsORC3a* in apomictic flowers at stage I arbitrarily set to 1. (This figure is available in colour at *JXB* online.)

PsORC3a copy (Aa) was constitutively and poorly expressed in apomictic flowers. The putative functional *PsORC3b* copy (Ab or Sb) was transcribed in both phenotypes and its expression level varied significantly between the different stages of sexual flower development and between apomictic and sexual flowers at the same developmental stages. In particular, *PsORC3b* expression increased greatly after anthesis in sexual flowers, whereas it remained low at the same stages in apomictic flowers (compare Sb with Ab in stages IV–VI). *PsORC3c* (either Ac or Sc) was poorly expressed in both phenotypes across over all reproductive stages, apparently lacking a time-related regulation.

To investigate the tissue specificity of the expression of the *PsORC3* genes, *in situ* hybridization (ISH) was undertaken on developing floral tissues of *P. simplex* and *P. notatum* (Fig. 4; Supplementary Figs S5–S7). Since probes specific to each *PsORC3* copy could not be developed, the signals detected in these experiments resulted from cross-hybridization of RNAs transcribed from all of them. In *P. simplex*, a detectable hybridization signal related to sense transcripts was evidenced in all developmental stages of sexual flowers. Signal was confined to the nucellus, megaspore mother cell, and functional megaspore before anthesis (Fig. 4A, E), to the nucellus, egg cells, and polar nuclei at anthesis (Fig. 4I; Supplementary Fig. S5A), and to the early endosperm in later stages (Fig. 4Q; Supplementary Fig. S7). Hybridization intensity was relatively low at pre-anthesis and anthesis stages, while it increased significantly in early endosperm, in full accordance with the qRT–PCR data. No hybridization signals were detected with the sense probe in the same organs and tissues (Fig. 4B, F, J, R). In apomictic flowers, *ORC3* expression showed the same tissue specificity as the sexual flowers in pre- and anthesis stages (Fig. 4C, G, K; Supplementary Fig. S5B), including the apospory initial cells (Fig. 4G). Conversely, the high expression detected in early sexual endosperm instead was nil in the corresponding apomictic endosperm (compare Fig. 4Q with S). Strikingly, the sense probe yielded evident hybridizing signals in the nucellus, egg cells, aposporic initial cells, and in polar nuclei of apomictic flowers (Fig. 4D, H, L). These are precisely the cells reproductively committed to apomixis, suggesting that the sense/antisense transcription of *PsORC3* might functionally mark the apomictic germ cell lineages in ovules of *P. simplex*. As a matter of fact, antisense transcripts were never detected in cells committed to sexual development; that is, the megaspore mother cell (Fig. 4D) and the functional megaspore (Fig. 4H), in a minimum of 15 ovules of the apomictic line scored. In order to verify the expression pattern of *PsORC3* transcripts in other species of *Paspalum*, ISH was carried out in flowers of the distantly related specie *P. notatum* at anthesis using the same probe as in *P. simplex*. Again, a clear sense signal was observed in sexual egg cells, polar nuclei, and nucellar tissue (Fig. 4M; Supplementary Fig. S5C) and a sense/antisense signals in the same cells of apomictic ovules (Fig. 4O, P; Supplementary Fig. S5C–L), suggesting that the sense/antisense transcription of *ORC3* is a general characteristic of the apomictic germ cell lineage in *Paspalum* species.

To investigate whether *PsORC3* is expressed in the male germ cell lineage, ISH was undertaken on apomictic and sexual developing anthers of *P. simplex* (Supplementary Fig. S6). Sense *ORC3* transcripts were highly expressed in the tapetum and archesporal cells (Supplementary Fig. S6A, C), moderately expressed in developing microsporocytes (Supplementary Fig. S6E, G), and not expressed in mature pollen grains in both sexual and apomictic anthers (Supplementary Fig. S6I, K). Again, antisense expression

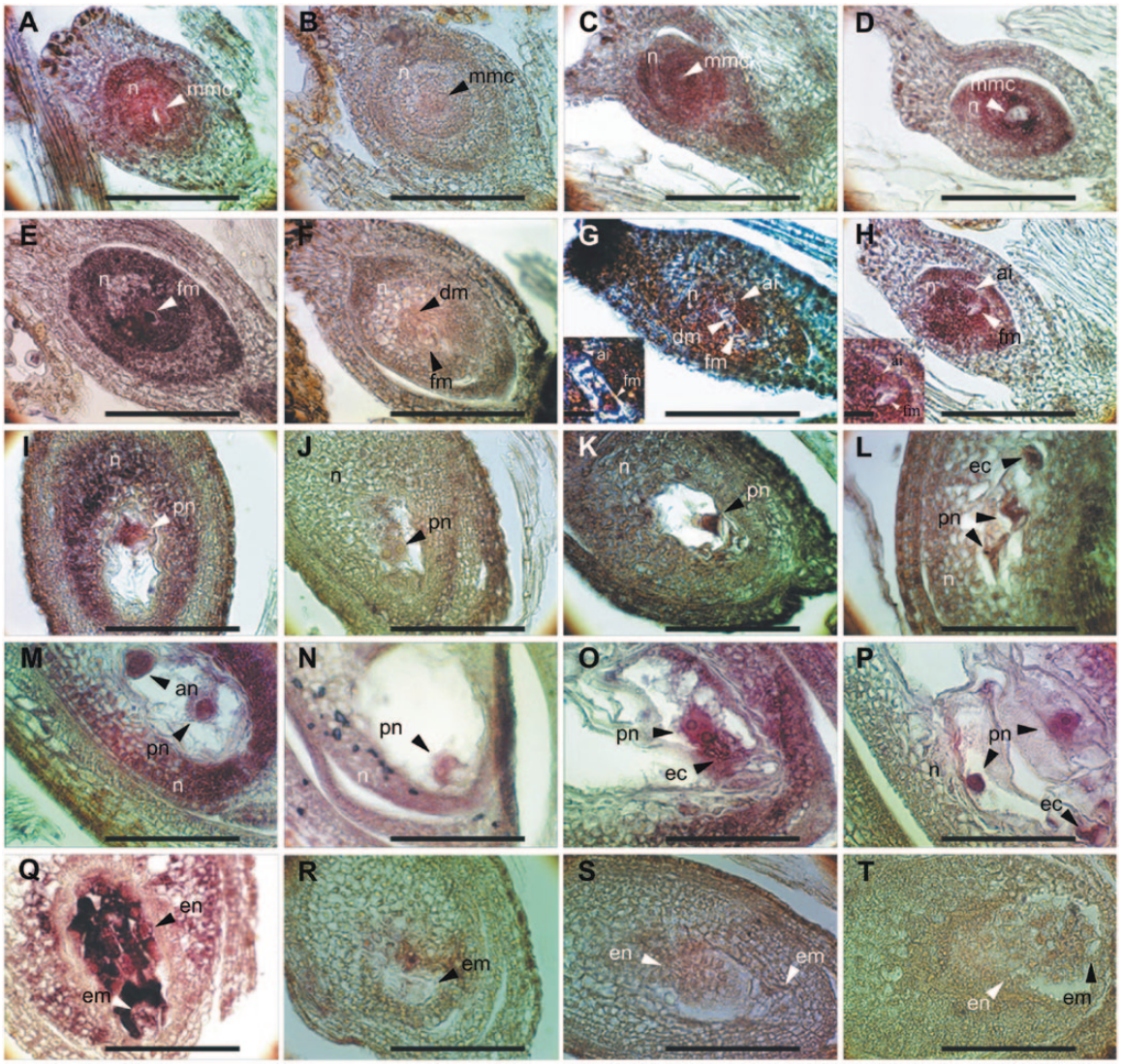

Fig. 4. *In situ* hybridization analysis of *PsORC3* transcripts in female reproductive tissues of sexual and apomictic *Paspalum* species. Ovules of sexual (A, B) and apomictic (C, D) genotypes of *P. simplex* at pre-meiosis (stage I). A strong hybridization signal was detected in the nucellus (n) and megaspore mother cell (mmc) with the antisense probe in both apomictic (A) and sexual (C) ovules and with the sense probe in the apomictic nucellus (D) only. No hybridization signal was observed with the sense probe in the sexual ovule (B) and in the megaspore mother cell of the apomictic ovule (D). Ovules of sexual (E, F) and apomictic (G, H) genotypes of *P. simplex* at post-meiosis/pre-gametogenesis (stage II). A strong hybridization signal was detected in the nucellus and the functional megaspore (fm) in sexual (E) and apomictic (G) ovules with the antisense probe, whereas no signal was detected with the sense probe in sexual ovules (F) or in the functional megaspore of apomictic ovules (H). Again, an evident hybridization signal was detected with the sense probe in the apomictic nucellus (H). Notably, both sense and antisense expression (higher magnification pictures in G and H, respectively) was detected in aposporic initial (ai) cells; dm, degenerating macrospores. Sections of sexual *P. simplex* (I, J) and *P. notatum* (M, N) ovules and apomictic *P. simplex* (K, L) and *P. notatum* (O, P) ovules at anthesis/end of gametogenesis (stage III). The antisense probe detected hybridization signal in the nucellus, polar nuclei (pn), and antipodal cells (an) in sexual (I, M) and in apomictic (K, O) ovules, where a positive signal was also detected in egg cells (ec). The sense probe detected a clear hybridization signal in the same cells of apomictic ovules (L, P), but not in the sexual ovules (J, N). Sections of sexual (Q, R) and apomictic (S, T) flowers 3 d after pollination (stage IV). A strong hybridization signal was evidenced with the antisense probe in early endosperm (en), but not in the embryo (em) of the early sexual seed (Q). No signal was detected with the same probe in the early apomictic seed (S). No hybridization signals were detected with the sense probe in either sexual (R) or apomictic (T) early seeds. Scale bars represent 100 μm. (This figure is available in colour at *JXB* online.)

was detected in developing cells of apomictic anthers only (Supplementary Fig. S6D, H). Water controls showed an absence of signals in all experiments (not shown).

To sum up, *PsORC3* is expressed in female and male germ cell lineages of both apomictic and sexual flowers as sense transcripts, whereas both sense and antisense strands are expressed in apomictic germ cells only. The putative functional gene is expressed in reproductively committed cells and tissues, mainly in early endosperm of sexual flowers, while it is markedly down-regulated in the same tissues of apomictic flowers.

Reverse genetics

To investigate the possible function of *PsORC3* and correlate it to its expression pattern in *Paspalum* species., the

phenotype of rice and Arabidopsis mutants defective for the homologous genes (LOC_Os10g26280 and At5G16690, respectively) were studied. In both species, this gene exists as a single-copy locus. Analysis of rice mutants included three insertion lines: Ne9014, Ng1015, and RdSpm2126B. In none of these lines were the transposons inserted within exons. The line Ne9014 had the insertion at nine nucleotides downstream of the ninth exon, Ng1015 at 53 bp downstream of the 12th exon, and RdSpm2126B at 234 bp downstream of the stop codon (Fig. 5A). Neither homozygous (*orc3*) nor heterozygous (*orc3*/+) individuals for insertion of lines Ne9014 and RdSpm2126B were phenotypically different from the WT for both vegetative and reproductive features. Furthermore, RT–PCR of flowers did not show differences for *OsORC3* expression among these genotypes (not shown). Conversely, although homozygous mutants of line Ng1015 were fully viable as shown by the Mendelian segregation of its selfed progeny into the expected 1(*orc3*):2(*orc3*/+):1(WT) ratio (Fig. 5B), they showed several vegetative and reproductive abnormalities (Fig. 6). The overall vegetative development of the *orc3* mutants was delayed and plants were weaker for almost all the vegetative structures compared with the WT. Mutants were smaller than the WT (Fig. 6A, B), had roots with a 'curly-like' phenotype (Fig. 6E, F), and the macrodissected flowers showed shorter stamen filaments (Fig. 6G, H). Furthermore, they showed necrotic lesions on paleas and lemmas (Fig. 6C, D) that were enhanced when plants were grown at 5–8 ºC below their optimal growth temperature. Although pollen stained with acetocarmine was viable (not shown), homozygous mutants were almost completely sterile, indicating that sterility affected female development (Table 1). To investigate whether sterility was due to gametophytic or sporophytic abnormalities, a comparative histological analysis of ovules of *orc3* and WT plants was undertaken at anthesis and 8–10 d after anthesis. WT ovules showed a single regular eight-nucleated embryo sac of *Polygonum* type at anthesis (Fig. 6J) and a well-developed embryo and endosperm 10 d after anthesis (Fig. 6L). Conversely, although the female gametophyte development in mutants was similar to that of the WT (Fig. 6I), the fertilized ovules were completely empty 10 d after anthesis, showing arrested embryos and endosperm reduced to a thin cell layer (Fig. 6K). The few developing endosperm cells observed 3–4 d after anthesis showed enlarged nuclei compared with the WT (Fig. 6M, N). This phenotype was specific to the *orc3* mutant, while *orc3*/+ was identical to the WT (not shown). Remarkably, the occurrence of the female sterile phenotype correlated with a dramatic decrease of *ORC3* gene expression (Fig. 5C). These results indicate that the mutation of the *ORC3* gene in the insertion line Ng1015 causes partial silencing of the target gene and induces a recessive maternal-effect embryo and/or endosperm lethality (i.e. a mutation that has no effect on embryo or endosperm viability when the homozygous mutant embryo develops within a heterozygous sporophyte, whereas it is lethal when the mutant embryo develops in a homozygous mutant environment; Ray *et al.*, 1996).

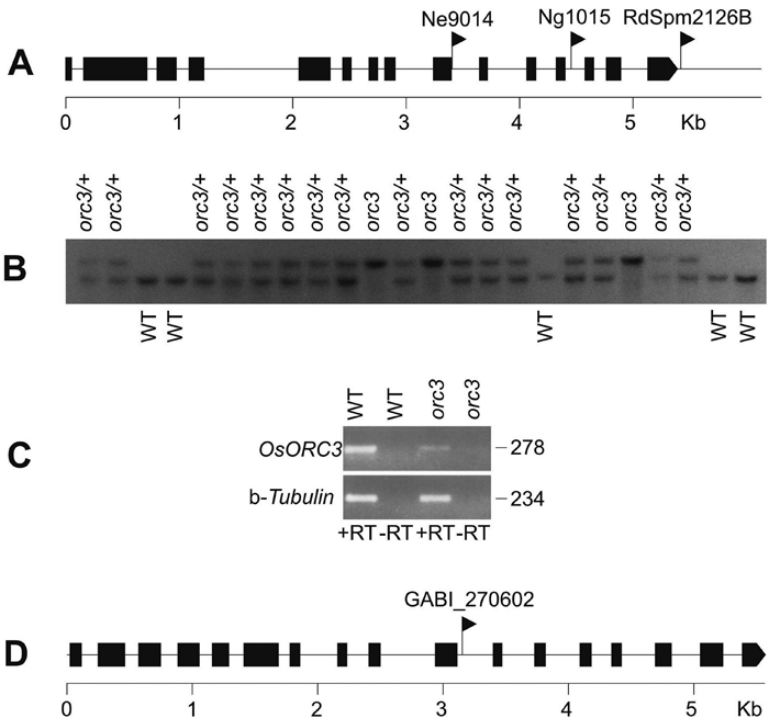

Fig. 5. The *orc3* mutants in rice and Arabidopsis. Predicted gene structure of *OsORC3* (A) and *AtORC3* (D); flags indicate the positions of the transposon (Tos17 for Ne9014 and Ng1015 and dSpm for RdSpm2126B lines) and T-DNA (for GABI_270602) insertions. (B) Southern blot analysis of *Xba*I-digested DNAs of a self-pollinated progeny from a heterozygous individual for the transposon insertion of the line Ng1015: *orc3*, *orc3*/+, and WT indicate homozygous, heterozygous, and wild-type individuals for the insertion, respectively. (C) RT–PCR analysis of the *OsORC3* gene in the Ng1015 rice mutant line at anthesis. +RT and –RT, indicate the presence or absence of reverse transcriptase in the reaction mixture. Amplicon size is expressed in bp.

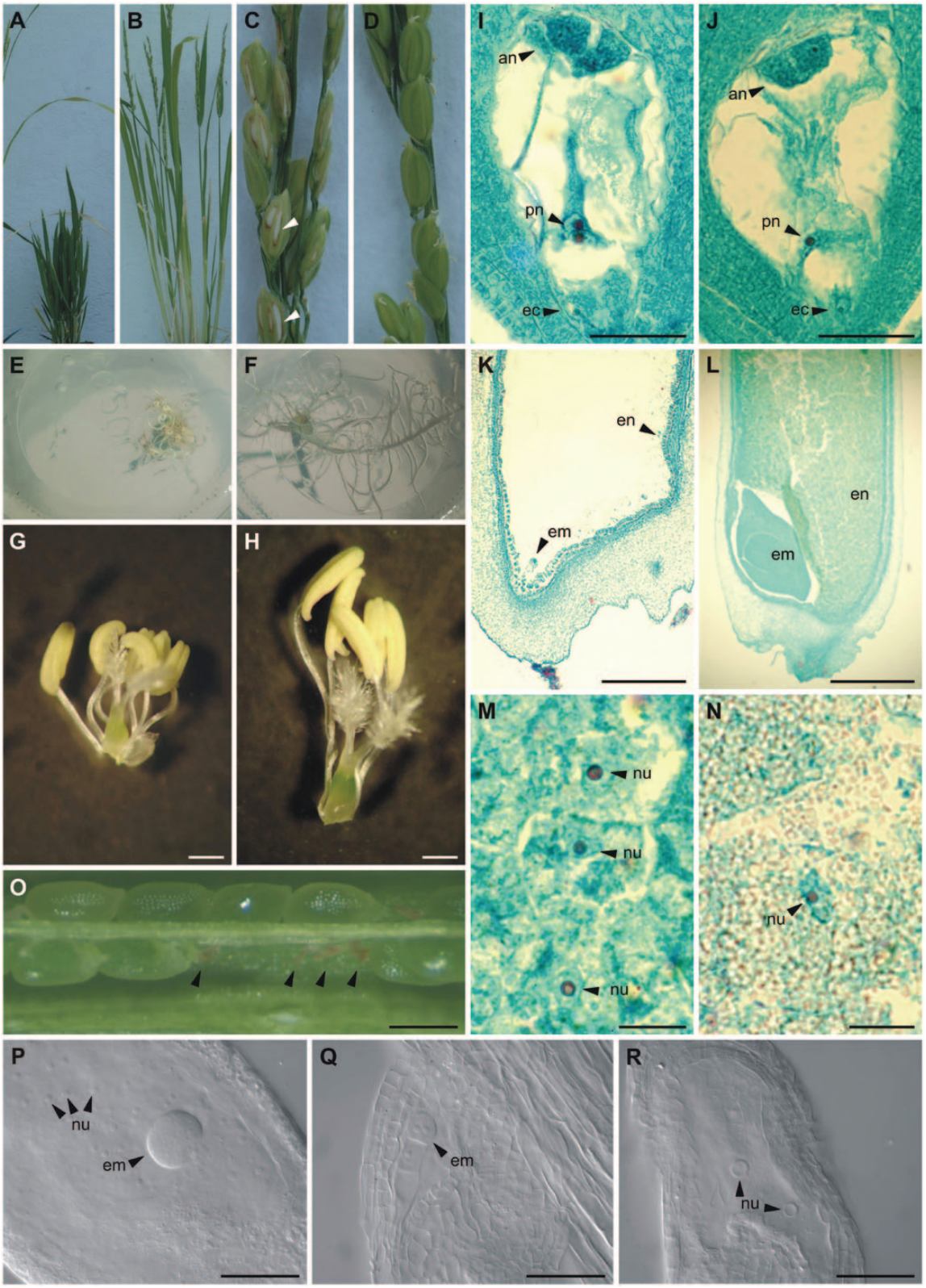

Fig. 6. Vegetative and reproductive abnormalities of rice Ng1015 (A–N) and Arabidopsis GABI_270602 (O–R) mutant lines for *ORC3*. Delayed vegetative development and plant weakness of *orc3* (A) compared with the WT (B), caryopses exhibiting necrotic lesions (arrowheads, C) not present in the WT (D), 'curly-like' mutant root phenotype compared with the WT (E and F, respectively), and shorter stamen filaments compared with the WT (G and H, respectively). Thin sectioned ovules of *orc3* (I) and the WT (J) at anthesis showing normal *Polygonum*-type embryo sacs. Seed of *orc3* at 10 d after pollination (K) showing an aborted embryo and a single cell layer endosperm compared with a normal WT seed (L). Endosperm cells with enlarged nuclei (nu) in *orc3* (M) compared with the WT (N). Siliques of *orc3*/+ showing abortive seeds (arrowheads, O), cleared seeds of the WT (P), and the *orc3* lethal phenotype (Q, R) of the *A. thaliana* GABI_270602 line. Arrowheads point to a two-cell arrested mutant embryo in (Q) and enlarged endosperm nuclei in (R) compared with those detected in the WT (P). Acronyms are as the same as those reported in Fig. 4 unless otherwise indicated. Scale bars represent: 10 μm in P, Q, and R; 20 μm in M and N; 40 μm in I and J; 500 μm in K, L, and O; and 1 mm in G and H. (This figure is available in colour at *JXB* online.)

Table 1. *Aborted rice seeds in progeny of 10 orc3/+ heterozygous mutants* for ORC3 *in the Ng1015 line*

Genotype	*n*	Comparisons	% of aborted seeds	Significance
WT	206	WT versus *orc3/+*	42% versus 62%	NS ($P<0.05$)
orc3/+	368	WT versus *orc3*	42% versus 99%	*** ($P<0.05$)
orc3	189	*orc3* versus *orc3/+*	62% versus 99%	*** ($P<0.05$)

To verify whether lesions on the *ORC3* gene induced a similar phenotype in Arabidopsis, a T-DNA insertion line (GABI_270G02) for the *AtORC3* gene (At5G16690) was analyzed. Similarly to the rice mutant lines, the T-DNA was inserted 40 bp downstream the 10th exon (Fig. 5D). Siliques of heterozygous plants for the mutation, containing 907 seeds in total, showed 18% aborted seeds (Fig. 6O), a percentage significantly higher than the WT (2% in a total of 850 seeds) and close to, even though not strictly consistent with, the proportion of 25% expected for a sporophytic lethal phenotype. The PCR screening for the T-DNA insertion yielded only heterozygous and WT plants at an approximate proportion of 1:1 (38 *orc3*/+:44 WT), that was significantly different from the 2:1 heterozygous:WT ratio expected for a sporophytic lethal mutation. Probably, the disruption of the *AtORC3* gene induced a sporophytic embryo lethality perhaps coupled with poor transmission of the mutated allele. This lethal phenotype was specific of the gynecium, as the pollen appeared viable (not shown). To investigate at which stage of embryogenesis *orc3* mutants were arrested, embryo development was studied in *orc3*/+ siliques after self-pollination. In the same silique, viable seeds showed either globular or heart stage embryos (Fig. 6P), whereas aborted seeds held embryos arrested at the 2- to 4-cell stage (Fig. 6Q). Furthermore, abnormal enlarged endosperm nuclei were observed in mutant seeds (Fig. 6R), suggesting an abnormal endoreduplication. Apart from the Ng1015 line, the Arabidopsis GABI_270G02 mutant did not show any maternal effect on embryo lethality. This could be due to the fact that unlike in rice, where *OsORC3* expression was high in flowers (Chen *et al.*, 2013), the same gene was poorly expressed in Arabidopsis inflorescences (Collinge *et al.*, 2004), perhaps making the level of its mRNAs below the threshold level necessary to exert any maternal control over embryo and/or endosperm development.

We therefore conclude that loss of function of the *ORC3* gene in both rice and Arabidopsis is causative of a recessive sporophytic seed-lethal phenotype as a consequence of embryo and/or endosperm abortion at a very early stage of development.

Discussion

The initiation of DNA replication in eukaryotic cells is a highly co-ordinated process linked to chromatin organization (Bell, 2002). The protein array made up of ORC, CDC6, CDT1, and MCM2-7, assembled at early G_1 phase and known as the pre-RC, makes chromatin competent for DNA replication (Bielinsky and Gerbi, 2001; Stoeber *et al.*, 2001; DePamphilis, 2003). Mutations in *ORC* genes result in cells arresting at the G_1/S transition (Bell, 2002), although in *Drosophila* (Loupart *et al.*, 2000) and yeast (Bell *et al.*, 1993) some cells can progress to the M-phases where they arrest and die. Moreover, individual ORC subunits can have additional functions in many cellular processes, often functioning to co-ordinate the initiation of DNA replication with essential cell cycle activities (Scholefield *et al.*, 2011). These functions are obviously related to cell proliferation and differentiation, and thus support a role in the basic processes of apomixis. Plant homologs of each of the six subunits of ORC proteins have been described in rice (Mori *et al.*, 2005; Shultz *et al.*, 2007), Arabidopsis (Masuda *et al.*, 2004), and, with the exception of subunit 6, in maize (Witmer *et al.*, 2003). Analysis of subunit inter-relationships within the ORC complex showed that ORC3 interacts strongly with ORC2 in rice (Tan *et al.*, 2013), Arabidopsis (Masuda *et al.*, 2004), and maize (Witmer *et al.*, 2003), and that the ORC3–ORC2 interaction is well conserved across taxa (Carpenter *et al.*, 1996; Pinto *et al.*, 1999; Vashee *et al.*, 2001). In particular, the ORC3 subunit plays a central role in the complex assembly in maize (Witmer *et al.*, 2003) as well as in the maintenance of complex association in Arabidopsis (Masuda *et al.*, 2004), thereby allowing the pre-RC machinery to function properly. A further confirmation of the strict ORC2/ORC3 functional relationship in Arabidopsis derives from the similarity of the enlarged endosperm nuclei mutant phenotype observed for the two genes [compare Fig. 6R of this study for *orc3* with fig. 2F of Collinge *et al.* (2004) for *orc2*].

Our results show that an *ORC3* (-like) pseudogene is genetically linked to apomixis, and its presence is associated with the down-regulation of its functional homolog (*PsORC3b*) during all stages of apomictic seed formation in *P. simplex*. Moreover, the inactivation of its ortholog in rice and Arabidopsis causes failure of endosperm development and arrest of embryo development at early stages.

Apomictic reproduction in *Paspalum* is controlled by a single complex dominant locus (ACR) that confers nearly 100% apospory, epigenetically controlled parthenogenesis (Podio *et al.*, 2014a), and the capacity to form endosperm to deviate from the canonical 2(maternal):1(paternal) ratio to the maternal excess 4(m):1(p) genome contribution ratio (Ortiz *et al.*, 2013). These three apomixis components are always inherited together as a linkage block, and no recombination events among them have been documented in the genus to date. Nevertheless, comparative mapping of the ACR showed an among-species moderate extent of recombination that screened out genes which were dispensable for apomictic reproduction and allowed the identification of a relatively narrow region that is conservatively linked to apomixis across distantly related species (Pupilli *et al.*, 2004; Hojsgaard *et al.*, 2011). This region is syntenic to the telomeric part of the long arm of chromosome 12 of rice that underwent both large- (inversion and/or translocation) and small-scale (small indels and/or point mutations) rearrangements when *Paspalum* and rice diverged from their common ancestor (Calderini *et al.*, 2006). However, interruption of colinearity through migration of genes from other locations appears to be the rule

rather than the exception (Calderini *et al.*, 2006), indicating that both chromosome segment rearrangements and gene migration could have acted synergistically to trigger apomictic reproduction in *Paspalum*. The rice homolog of *PsORC3*, physically located on rice chromosome 10, is probably one of the migrating genes that had been retained in the ACR during *Paspalum* evolution together with others that contributed to the establishment of pseudogamous apomixis in this genus.

Molecular characterization and expression analyses of *PsORC3* showed that three *ORC3* copies are present in *P. simplex*. Of these, *PsORC3b* that encodes a putative functional protein was up-regulated at anthesis and post-anthesis in the sexual genotypes and down-regulated in the same stages in apomicts. ISH experiments showed the presence of *PsORC3* transcripts in germ cell lineages and in early endosperm, but also revealed the presence of an antisense transcript only in the apomictic flowers, suggesting that it could be associated with the down-regulation of *PsORC3b* in the same flowers. Such transcripts were absent in the sexual female cell lineages of the apomictic flowers; that is, in the megaspore mother cell and functional megaspore, indicating that the antisense expression may reflect different roles of the *ORC3* genes in the two reproductive pathways. Roles of individual ORC subunits apart from the cell cycle machinery have been reported in Arabidopsis (de la Paz Sanchez and Gutierrez, 2009). In any case, the possibility to mark the apomictic cell lineages physiologically in ovaries provides functional evidence of the visionary perspective expressed by Harlan *et al.* (1964) according to which 'apomixis and sexual reproduction are not alternative modes of reproduction, either genetically or operationally, but are independent and simultaneous phenomena'. Therefore, apomixis and sexuality are not mutually exclusive in Mendelian terms but, particularly in the case of aposporous apomixis, the two reproductive modes co-exist in the same ovary and the prevalence of one mode over the other depends on as yet unknown post-meiotic factors. Because no *ORC3* transcripts were present in early apomictic endosperm, it is possible that a suppression mechanism based on antisense expression might be acting at this stage when the amount of sense target mRNA is higher than a threshold level that is reached only in early endosperm. Evidence of such mRNA dosage-dependent mechanisms of gene suppression mediated by sense–antisense complex formation is reported in yeasts (Raponi *et al.*, 2000; Raponi and Arndt, 2003).

Reverse genetics analysis of defective mutants in Arabidopsis and rice for *ORC3* showed that this gene has little (Arabidopsis) or no effect (rice) in female gametophyte development, whereas it plays a crucial role in endosperm and/or embryo development. The same role in post-fertilization development can be hypothesized in *P. simplex* as *PsORC3b* is up-regulated after fertilization in sexual flowers, whereas it is silenced in apomictic flowers. Therefore, the most probable role of *PsORC3* in apomictic reproduction should be inherent to specific post-fertilization events such as the development of: (i) a parthenogenetic embryo; and/or (ii) 4(m):1(p) maternal excess endosperm. As significant expression differences between apomictic and sexual flowers for *PsORC3* were detected only in early endosperm, we favor the hypothesis that this gene could play a role in the development of this organ in apomictic seeds. In *Paspalum* species, sexual strains (diploids and tetraploids) are sensitive to the 2m:1p genome ratio in the endosperm whereas apomictic strains display some degree of relaxation of this regulation (Quarin, 1999; Hojsgaard *et al.*, 2013). For example, tetraploid apomictic *P. notatum* strains showed the maximum reproductive efficiency in crosses with pollen from a tetraploid, giving a parental genome ratio in the endosperm of 4m:1p (Quarin, 1999) that is structurally equivalent to that derived from $4x \times 2x$ crosses, with maternal excess in the endosperm. In Arabidopsis, interploidy crosses in which the maternal genome in the endosperm exceeded the canonical 2m:1p ratio produced seeds with reduced size, whereas reciprocal crosses yielded abortive larger seeds (Scott *et al.*, 1998). Similar results were obtained in interploidy crosses in rice (Sekine *et al.*, 2013), and in maize (Leblanc *et al.*, 2002). Conversely, apomictic seeds with an excess of the maternal genome in the endosperm differ neither for seed size nor for viability from the sexual seeds in which the genomic ratio of 2m:1p is observed. Abrupt down-regulation of *PsORC3* in the early endosperm development of apomictic *P. simplex* could be related to the relaxation of the endosperm balance control, allowing proper development of maternal excess apomictic endosperm. Furthermore, the fact that *ORC* genes were down-regulated in maternal excess endosperm in Arabidopsis (Tiwari *et al.*, 2010) is an additional indication of the possible role of *ORC* genes in overcoming genome imbalance in endosperm. We hypothesize that in sexual *Paspalum*, in crosses generating the canonical 2m:1p ratio in the endosperm, *PsORC3*, as a key component of the *ORC* complex, is up-regulated to facilitate endosperm development perhaps through the action of a ploidy sensor gene. The maternally imprinted gene *MEDEA* (*MEA*) (Grossniklaus *et al.*, 1998; Kiyosue *et al.*, 1999) acts as a ploidy sensor in the endosperm of Arabidopsis (Erilova *et al.*, 2009), and a functional link between *MEA* and *ORC* genes has been proposed by Collinge *et al.* (2004). Conversely, in the endosperm of apomictic lines which show a maternal excess, the functional link between MEA and ORC in silencing the DNA replication complex can be bypassed, because ORC3 is already silenced and endosperm development is facilitated by an alternative as yet unknown route. However, conclusive evidence of the role of *ORC3* in controlling the development of apomictic endosperm needs to be gained by studying it in homologous agamic biological systems (i.e. species including both sexual and apomictic cytotypes) as only these have developed such alternative routes. Experiments are in progress in order to inactivate *ORC3* in tetraploid sexual *P. simplex* to use the resulting mutants as female parents in crosses with diploid pollinators to re-create the 4m:1p maternal excess typical of apomictic endosperm in a sexual context.

It has been proposed that apomixis is superimposed epigenetically over the sexual default state by the silencing action of two independent loci, LOA and LOP (Koltunow *et al.*, 2011), with a mechanism similar to that exercised by the gene cluster contained in the Y-chromosome to silence the female genes in dioecious plants (Pupilli and Barcaccia, 2012; Ortiz *et al.*, 2013). Then, as in many of the known Y-systems in which there are both silencer genes of female development

and positive acting genes able to promote the development of male structures, the ACR could carry silencers of sexuality and enhancers of apospory, parthenogenesis, and (in the case of pseudogamous apomicts) development of maternal excess endosperm. Here we report that a fundamental gene for sexual seed formation is down-regulated in apomictic genotypes probably because of the presence of its homologous pseudogene. Our work points to the presence of active mechanisms repressing sexuality in apomictic *P. simplex* in which *PsORC3* seems to play an active role. The elucidation of such mechanisms will be crucial to the success of breeding programs aimed at the introgression of apomixis into major crops by genetic engineering. In particular, the in-depth analysis of *PsORC3* could help to overcome not only the strict 2m:1p endosperm balance requirement that is a major bottleneck for the introgression of apomixis in major crops, but also the sterility due to maternal excess endosperm in interploidy crosses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. ClustalW alignment of the three *PsORC3* gene copies present in the *P. simplex* genome.

Figure S2. RFLP banding patterns of *Eco*RI DNA digests of apomictic and sexual plants of three *Paspalum* species hybridized with a probe developed on the *PsORC3* sequence.

Figure S3. ClustalW alignment of deduced protein sequences of *PsORC3b* and *PsORC3c* together with 12 *ORC3* sequences belonging to other plants.

Figure S4. Unrooted consensus tree generated by an alignment matrix between PsORC3b, PsORC3c, and 15 ORC3 protein sequences of different organisms.

Figure S5. *In situ* hybridization analysis of *PsORC3* transcripts in female reproductive tissues of sexual and apomictic *Paspalum* species at anthesis.

Figure S6. *In situ* hybridization analysis of *PsORC3* transcripts in male reproductive tissues of sexual and apomictic flowers of *P. simplex*.

Figure S7. *In situ* hybridization analysis of *PsORC3* transcripts in three contiguous cuts of an early seed of sexual *P. simplex*.

Table S1. Primers used in this study

Acknowledgements

This work was financed by the Ministero dell'Istruzione, dell'Università e della Ricerca, Italy (PRIN project, 2007LC7RBF_003), the Dipartimento di Scienze Bio-Agroalimentari del Consiglio Nazionale delle Ricerche, Italy (Laboratori Congiunti Bilaterali Internazionali del CNR, Prot. 0005651), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina (Project PIP,11220090100613), the Agencia Nacional de Promoción Científica y Tecnológica, Argentina (Projects PICT 2011-1269 and 2014-1080), and the Universidad Nacional de Rosario, Argentina (Project AGR189). F. Pupilli received a fellowship for a sabbatical visit from CONICET, and PK received a fellowship from the Department of Biotecnhology (Indian Biotech Overseas Associateship).

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research **25,** 3389–3402.

Bell SP. 2002. The origin recognition complex: from simple origins to complex functions. Genes and Development **16,** 659–672.

Bell SP, Dutta A. 2002. DNA replication in eukaryotic cells. Annual Review of Biochemistry **71,** 333–374.

Bell SP, Kobayashi R, Stillman B. 1993. Yeast origin recognition complex functions in transcription silencing and DNA replication. Science **262,** 1844–1849.

Bielinsky A-K, Gerbi SA. 2001. Where it all starts: eukaryotic origins of DNA replication. Journal of Cell Science **114,** 643–651.

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlach J. 2001. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. The Plant Cell **13,** 1499–1510.

Cáceres ME, Matzk F, Busti A, Pupilli F, Arcioni S. 2001. Apomixis and sexuality in *Paspalum simplex*: characterization of the mode of reproduction in segregating progenies by different methods. Sexual Plant Reproduction **14,** 201–206.

Cáceres ME, Pupilli F, Quarin CL, Arcioni S. 1999. Feulgen-DNA densitometry of embryo sacs permits discrimination between sexual and apomictic plants in *Paspalum simplex.* Euphytica **110,** 161–167.

Calderini O, Chang SB, de Jong H, *et al.* 2006. Molecular cytogenetics and DNA sequence analysis of an apomixis-linked BAC in *Paspalum simplex* reveal a non-pericentromere location and partial microcolinearity with rice. Theoretical and Applied Genetics **112,** 1179–1191.

Calderini O, Donnison I, Polegri L, Panara F, Thomas A, Arcioni S, Pupilli F. 2011. Partial isolation of the genomic region linked with apomixis in *Paspalum simplex*. Molecular Breeding **28,** 265–276.

Carpenter PB, Mueller PR, Dunphy WG. 1996. Role for a Xenopus Orc2-related protein in controlling DNA replication. Nature **379,** 357–360.

Chen X, Shi J, Hao X. 2013. *OsORC3* is required for lateral root development in rice. The Plant Journal **74,** 339–350.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research **31,** 3497–3500.

Collinge MA, Spillane C, Kohler C, Gheyselinck J, Grossniklaus U. 2004. Genetic interaction of an origin recognition complex subunit and the Polycomb group gene *MEDEA* during seed development. The Plant Cell **16,** 1035–1046.

Conner JA, Mookkan M, Huo H, Chae K, Ozias-Akins P. 2015. A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. Proceedings of the National Academy of Sciences, USA **112,** 11205–11210.

Corral JM, Vogel H, Aliyu OM, Hensel G, Thiel T, Kumlehn J, Sharbel TF. 2013. A conserved apomixis-specific polymorphism is correlated with exclusive exonuclease expression in premeiotic ovules of apomictic *Boechera* species. Plant Physiology **163,** 1660–1672.

de la Paz Sanchez M, Gutierrez C. 2009. *Arabidopsis* ORC1 is a PHD-containing H3K4me3 effector that regulates transcription. Proceedings of the National Academy of Sciences, USA **106,** 2065–2070.

DePamphilis ML. 2003. The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. Gene **310,** 1–15.

Erilova A, Brownfield L, Exner V, Rosa M, Twell D, Scheid OM, Hennig L, Köhler C. 2009. Imprinting of the Polycomb group gene *MEDEA* serves as a ploidy sensor in Arabidopsis. PLoS Genetics **5,** e1000663.

Felitti SA, Seijo JG, Gonzalez AM, Podio M, Laspina NV, Siena L, Ortiz JPA, Pessino SC. 2011. Expression of lorelei-like genes in aposporous and sexual *Paspalum notatum* plants. Plant Molecular Biology **77,** 337–354.

Finn RD, Bateman A, Clements J, *et al.* 2013. Pfam: the protein families database. Nucleic Acids Research **42,** D222–D230.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research **31,** 3784–3788.

Grossniklaus U, Vielle-Calzada J-P, Hoeppner MA, Gagliano WB. 1998. Maternal control of embryogenesis by *MEDEA*, a *Polycomb* group gene in *Arabidopsis*. Science **280,** 446–450.

Hand ML, Koltunow AMG. 2014. The genetic control of apomixis. Genetics **197,** 441–450.

Hanna WW. 1995. Use of apomixis in cultivar development. Advances in Agronomy **54,** 333–350.

Harlan JR, Brooks MH, Borgaonkar DS, de Wet JMJ. 1964. Nature and inheritance of apomixis in *Bothriochloa* and *Dichanthium*. Botanical Gazette **125,** 41–46.

Hojsgaard DH, Martínez EJ, Acuña CA, Quarin CL, Pupilli F. 2011. A molecular map of the apomixis-control locus in *Paspalum procurrens* and its comparative analysis with other species of *Paspalum*. Theoretical and Applied Genetics **123,** 959–971.

Hojsgaard DH, Martínez EJ, Quarin CL. 2013. Competition between meiotic and apomictic pathways during ovule and seed development results in clonality. New Phytologist **197,** 336–347.

Kiyosue T, Ohad N, Yadegari R, *et al*. 1999. Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis.* Proceedings of the National Academy of Sciences, USA **96,** 4186–4191.

Kleinboelting N, Huep G, Kloetgen A, Viehoever P, Weisshaar B. 2012. GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. Nucleic Acids Research **40,** D1211–D1215.

Koltunow AM, Johnson SD, Rodrigues JCM, *et al*. 2011. Sexual reproduction is the default mode in apomictic *Hieracium* subgenus *Pilosella*, in which two dominant loci function to enable apomixis. The Plant Journal **66,** 890–902.

Kumar CS, Wing RA, Sundaresan V. 2005. Efficient insertional mutagenesis in rice using the maize En/Spm elements. The Plant Journal **44,** 879–897.

Laspina NV, Vega T, Seijo JG, *et al*. 2008. Gene expression analysis at the onset of aposporous apomixis in *Paspalum notatum*. Plant Molecular Biology **67,** 615–628.

Leblanc O, Pointe C, Hernandez M. 2002. Cell cycle progression during endosperm development in *Zea mays* depends on parental dosage effects. The Plant Journal **32,** 1057–1066.

Liu C-M, Meinke DW. 1998. The titan mutants of *Arabidopsis* are disrupted in mitosis and cell cycle control during seed development. The Plant Journal **16,** 21–31.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25,** 402–408.

Loupart M-L, Krause SA, Heck MMS. 2000. Aberrant replication timing induces defective chromosome condensation in *Drosophila* ORC2 mutants. Current Biology **10,** 1547–1556.

Mancini M, Woitovich N, Permingeat HR, Podio M, Siena LA, Ortiz JPA, Pessino SC, Felitti SA. 2014. Development of a modified transformation platform for apomixis candidate genes research in *Paspalum notatum* (bahiagrass). In Vitro Cell Developmental Biology-Plant **50,** 412–424.

Marimuthu MP, Jolivet S, Ravi M, *et al*. 2011. Synthetic clonal reproduction through seeds. Science **331,** 876.

Martínez EJ, Urbani MH, Quarin CL, Ortiz JPA. 2001. Inheritance of apospory in bahiagrass, *Paspalum notatum*. Hereditas **135,** 19–25.

Masuda HP, Ramos GBA, de Almeida-Engler J, Cabral LM, Coqueiro VM, Macrini CMT, Ferreira PCG, Hemerly AS. 2004. Genome based identification and analysis of the pre-replicative complex of *Arabidopsis thaliana*. FEBS Letters **574,** 192–202.

Mau M, Corral JM, Vogel H, Melzer M, Fuchs J, Kuhlmann M, de Storme N, Geelen D, Sharbel TF. 2013. The conserved chimeric transcript UPGRADE2 is associated with unreduced pollen formation and is exclusively found in apomictic *Boechera* species. Plant Physiology **163,** 1640–1659.

Miyao A, Tanaka K, Murata K, Sawaki H, Takeda S, Abe K, Shinozuka Y, Onosato K, Hirochika H. 2003. Target site specificity of the Tos17 retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome. The Plant Cell **15,** 1771–1780.

Mori Y, Yamamoto T, Sakaguchi N, Ishibashi T, Furukawa T, Kadota Y, Kuchitsu K, Hashimoto J, Kimura S, Sakaguchi K. 2005. Characterization of the origin recognition complex (ORC) from a higher plant, rice (*Oryza sativa* L.). Gene **353,** 23–30.

Motulsky HJ. 2003. Graphpad InStat® version 3.00 for Windows 95, Graphpad Software . San Diego, CA, www.graphpad.com.

Nogler GA. 1984. Gametophytic apomixis. In: Johri BM, ed. Embryology of angiosperms . Berlin: Springer, 475–518.

Ortiz JPA, Quarin CL, Pessino SC, Acuña C, Martínez EJ, Espinoza F, Hojsgaard DH, Sartor ME, Cáceres ME, Pupilli F. 2013. Harnessing apomictic reproduction in grasses: what we have learned from *Paspalum*. Annals of Botany **112,** 767–787.

Ozias-Akins P, van Dijk PJ. 2007. Mendelian genetics of apomixis in plants. Annual Review of Genetics **41,** 509–537.

Pessino SC, Espinoza F, Martínez EJ, Ortiz JPA, Valle EM, Quarín CL. 2001. Isolation of cDNA clones differentially expressed in flowers of apomictic and sexual *Paspalum notatum*. Hereditas **134,** 35–42.

Pinto S, Quintana DG, Smith P, *et al*. 1999. *latheo* encodes a subunit of the origin recognition complex and disrupts neuronal proliferation and adult olfactory memory when mutant. Neuron **23,** 45–54.

Podio M, Cáceres ME, Samoluk SS, Seijo JG, Pessino SC, Ortiz JPA, Pupilli F. 2014*a*. A methylation status analysis of the apomixis-specific region in *Paspalum* spp. suggests an epigenetic control of parthenogenesis. Journal of Experimental Botany **65,** 6411–6424.

Podio M, Felitti SA, Siena LA, Delgado L, Mancini M, Seijo JG, González AM, Pessino SC, Ortiz JPA. 2014*b*. Characterization and expression analysis of *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) genes in sexual and apomictic *Paspalum notatum*. Plant Molecular Biology **84,** 479–495.

Polegri L, Calderini O, Arcioni S, Pupilli F. 2010. Specific expression of apomixis-linked alleles revealed by comparative transcriptomic analysis of sexual and apomictic *Paspalum simplex* Morong flowers. Journal of Experimental Botany **61,** 1869–1883.

Pupilli F, Barcaccia G. 2012. Cloning plants by seeds: inheritance models and candidate genes to increase fundamental knowledge for engineering apomixis in sexual crops. Journal of Biotechnology **159,** 291–311.

Pupilli F, Labombarda P, Cáceres ME, Quarin CL, Arcioni S. 2001. The chromosome segment related to apomixis in *Paspalum simplex* is homoeologous to the telomeric region of the long arm of rice chromosome 12. Molecular Breeding **8,** 53–61.

Pupilli F, Martínez EJ, Busti A, Calderini O, Quarin CL, Arcioni S. 2004. Comparative mapping reveals partial conservation of synteny at the apomixis locus in *Paspalum* spp. Molecular Genetics and Genomics **270,** 539–548.

Quarin CL. 1999. Effect of pollen source and pollen ploidy on endosperm formation and seed set in pseudogamous apomictic *Paspalum notatum*. Sexual Plant Reproduction **11,** 331–335.

Raponi M, Arndt GM. 2003. Double-stranded RNA-mediated gene silencing in fission yeast. Nucleic Acids Research **31,** 4481–4489.

Raponi M, Atkins D, Dawes I, Arndt G. 2000. The influence of antisense gene location on target gene suppression in the fission yeast *Schizosaccharomyces pombe*. Antisense and Nucleic Acid Drug Development **10,** 29–34.

Ray S, Golden T, Ray A. 1996. Maternal effects of the *short integument* mutation on embryo development in *Arabidopsis*. Developmental Biology **180,** 365–369.

Remus D, Diffley JF. 2009. Eukaryotic DNA replication control: lock and load, then fire. Current Opinion in Cell Biology **21,** 771–777.

Sambrook J, Fritsch EF, Maniatis T. 1988. Molecular cloning: a laboratory manual . Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Scholefield G, Veening J-W, Murray H. 2011. DnaA and ORC: more than DNA replication initiators. Trends in Cell Biology **21,** 188–194.

Scott RJ, Spielman MJ, Bailey J, Dickinson HG. 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana.* Development **125,** 3329–3341.

Sekine D, Ohnishi T, Furuumi H, Ono A, Yamada T, Kurata N, Kinoshita T. 2013. Dissection of two major components of the post-zygotic hybridization barrier in rice endosperm. The Plant Journal **76,** 792–799.

Shultz RW, Tatineni VM, Hanley-Bowdoin L, Thompson WF. 2007. Genome-wide analysis of the core DNA replication machinery in the higher plants *Arabidopsis* and rice. Plant Physiology **144,** 1697–1714.

Siena LA, Ortiz JPA, Leblanc O, Pessino SC. 2014. PnTgs1 expression during reproductive development supports a role for RNA methyltransferases in the aposporous pathway. BMC Plant Biology **14,** 297–307.

Stoeber K, Tlsty TD, Happerfield L, Thomas GA, Romanov S, Bobrow L, Williams ED, Williams GH. 2001. DNA replication licensing and human cell proliferation. Journal of Cell Science **114,** 2027–2041.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution **30,** 2725–2729.

Tan D, Lv Q, Chen X, Shi J, Ren M, Wu P, Mao C. 2013. Interactions among rice ORC subunits. Plant Signaling and Bahavior **8,** e25007.

Tiwari S, Spielman M, Schulz R, Oakey RJ, Kelsey G, Salazar A, Zhang K, Pennell R, Scott RJ. 2010. Transcriptional profiles underlying parent-of-origin effects in seeds of *Arabidopsis thaliana*. BMC Plant Biology **10,** 72.

Vashee S, Simancek P, Challberg MD, Kelly TJ. 2001. Assembly of the human origin recognition complex. Journal of Biological Chemistry **276,** 26666–26673.

Vielle-Calzada JP, Crane CF, Stelly DM. 1996. Apomixis: the asexual revolution. Science **274,** 1322–1323.

Witmer XH, Alvarez-Venegas R, San-Miguel P, Danilevskaya O, Avramova Z. 2003. Putative subunits of the maize origin of replication recognition complex ZmORC1–ZmORC5. Nucleic Acids Research **31,** 619–628.