DEVELOPING BIOTECHNOLOGICAL TOOLS TO STUDY APOMIXIS IN *BRACHIARIA*

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Apomixis is a trait with a single genetic control (reviewed in OZIAS-AKINS, 2006). Therefore, can be manipulated through the conventional techniques of plant genetic improvement as well as genetic engineering techniques. Because of the prospect of controlling apomictic reproduction, interest on this trait has increased recently. The main interest in uncovering the apomixis pathway is the possibility of control and transfer of the trait to cultivated plants where apomixis does not occur naturally. Consequences of this strategy have been discussed by many authors (BASHAW, 1980; ASKER; JERLING, 1992; KOLTUNOW, 1993; CARNEIRO; DUSI, 2002; SPILLANE et al., 2004) as follows: fixation of hybrid heterosis vigor through seed propagation; the possibility of propagating and storing currently vegetatively-propagated plants by seeds; simplification of commercial hybrid seed production with consequent reduction in cost; simplification of breeding programs and consequently increasing the number of cultivars adapted to specific environments.

Apomixis and Amphimixis - types of reproduction

Apomixis is a process that occurs in the female part of the flower, in the ovary and more specifically in the ovule. Apomixis has a strong connection with the sexual reproduction pathway. Currently, it is proposed that apomixis is the result of a rearrangement of the sexual pathway (revised in TUCKER; KOLTUNOW, 2009). Therefore the comparison of both apomixis and sexuality is the obvious way to interpret this special phenomenon.

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Amphimixis

In amphimixis or sexual reproduction, meiotic divisions provide the reduction of chromosome number to form a reduced gametophyte, and embryos developed from zygotes are a result of the fusion of reduced male and female gametes. Three main steps are recognized: the development of the male and female gametes inside differentiated structures, pollen grains and embryo sac, the gametophytes; the event of double fertilization and the embryo development.

The most common embryo sac in angiosperm is the meiotic embryo sac of the Polygonum type (WILLEMSE; VAN WENT, 1984). It is a result of chromosome number reduction through meiosis. This embryo sac originates from an unreduced (2n) sub epidermal cell of the ovule, the archesporial cell that turns into the megaspore mother cell. During the megasporogenesis this cell undergoes two meiotic divisions to form a linear tetrad of reduced cells, the megaspores (n). Three megaspores close to the micropyle degenerate while the chalazal one survives as a functional megaspore. During the megagametogenesis the nucleus of the functional megaspore undergoes three successive mitoses to form an embryo sac with eight reduced nuclei (n). After cellularization seven cells are formed: three antipodal cells at the chalazal pole; a central cell containing two polar nuclei; two synergids and an egg cell at the micropylar pole forming the egg apparatus. The polar nuclei fuse to form a diploid central cell nucleus. During anthesis double fertilization occurs, when one of the male gametes (n) fuses with the egg cell (n) to form the zygote nucleus (2n) and the other male gamete fuses with the polar nucleus (2n) and will further form the endosperm (3n). The embryo develops from the zygote using the endosperm as a source of nutrients. Thus, the progeny resulting from the sexual reproduction is genetically different from their progenitors.

Apomictic reproduction

In apomictic reproduction meiosis is partially or completely avoided and fertilization with the fusion of the male and female gametes does not occur. Embryos carrying only the maternal set of chromosomes are formed from unreduced cells in one of the mechanisms described here. Apomixis can be sporophytic or gametophytic (NOGLER 1984; ASKER; JERLING, 1992). Sporophytic apomixis or adventitious embryony or even nucellar embryony is the result of the direct development of somatic cells of the nucellus or inner

integument into embryos by successive mitoses (LAKSHMANAN; AMBEGAOKAR, 1984). Gametophytic apomixis is characterized by apomeiosis (NOGLER 1984) and can occur either by diplospory or apospory (NOGLER 1984; ASKER; JERLING, 1992). In diplospory an unreduced embryo sac develops from the megaspore mother cell that bypasses or interrupts meiosis and undergoes mitoses. Autonomous embryos and often autonomous endosperm are formed (TUCKER; KOLTUNOW, 2009). In apospory unreduced embryo sacs are formed from somatic cells of the nucellus that differentiate and undergo two or three mitotic divisions while the meiotic development of the megaspore mother cell takes place or is interrupted. Embryos are formed autonomously and endosperm may or may not need fertilization to develop. If the meiotic development occurs, it is possible to find in the same ovary aposporic embryo sacs side by side with the sexuals. Many embryo sacs can be present in one ovule. *Brachiaria,* as many of the apomictic grasses, presents the apospory type of apomixis.

Reproduction by apomixis has been reported in many species of the Poaceae family, many of which are forage grasses. Grasses of the genus *Brachiaria* (Trin.) Griseb. are included among 100 species that occur mainly in Africa (RENVOIZE et al., 1996; DAHLGREN et al., 1985). A number of these species are widely cultivated in tropical areas of Cerrado in Brazil with economical and environmental importance. Two essentially apomictic species, *Brachiaria brizantha and B. decumbens,* are responsible for the majority of the pasture area in this region.

Brachiaria reproduction

The inflorescence of *Brachiaria* is a panicle composed of 2 to 5 racemes that support the spikelets placed in two series on the raceme. Two flowers develop in each spikelet, a male flower and a hermaphrodite one (NDIKUMANA, 1985). The male flower has three anthers and seems to develop normally releasing pollen grains during anthesis. The hermaphrodite flower has three anthers and one ovule.

Some species of *Brachiaria* present both sexual and apomictic modes of reproduction. Plants are classified as sexual when they have meiotic embryo sacs in their ovaries and few empty and sterile ovaries. Apomictic plants have mainly aposporic embryo sacs, that can be single or multiple, and meiotic embryo sacs in variable degrees, indicating that apomixis is probably facultative (VALLE, 1990).

Meiotic embryo sacs are of the Polygonum type, with late proliferation of up to 12 antipodal cells (GOBBE et al., 1982; LUTTS et al., 1994). Aposporic embryo sacs are of the Panicum type and have four unreduced nuclei distributed in one pole that after cellularization correspond to an egg apparatus and a central cell. Apomixis is pseudogamic and need pollination and fertilization of the central cell to develop the endosperm (NGENDAHAYO, 1988; ALVES et al., 2001). Two major steps of regulation of apospory are recognized: the differentiation of the apospore initials and the autonomous development of the unreduced egg cell and endosperm development. Currently it is believed that only a few genes are responsible for controlling apospory. A model of one dominant gene regulating apospory was suggested for *Brachiaria* (NDIKUMANA, 1985; VALLE; SAVIDAN, 1996).

Apomixis in *Brachiaria,* as reported for many other genera, is associated with polyploidy. In most cases apomicts are polyploids, usually tetraploid and corresponding sexuals are diploid. Alternatively, the ploidy of apomicts is higher as in *B. humidicola* in which the sexual is tetraploid and the apomictic is hexaploid. In *B. ruziziensis* (SWEENE et al., 1981; GOBBE et al., 1981 1982) and *B. brizantha* (PINHEIRO et al., 2000; ARAÚJO et al,. 2005) the duplication of the chromosome number of sexual diploid kept their sexuality, in contrast to the observations in *Paspalum* that duplicating sexual diploids generated facultative apomict (QUARIN et al., 2001). Breeding of *Brachiaria* as well as in other natural apomicts is very difficult due to the difference in ploidy of sexuals and apomicts that make crosses not viable (VALLE et al., 2004). For many years, the improvement of *Brachiaria* consisted in the selection of superior genotypes among the naturally existing ones (MILLES; VALLE, 1996; ARAÚJO et al., 2008). The cultivated *Brachiaria* corresponds to only very few well adapted varieties that are predominantly apomictic and therefore uniform; it has the characteristic of an extensive monoculture that implies in great economical risk. Improved cultivars with forage quality and also resistant to pest and diseases are required. In this direction, and also for the study of apomixis inheritance in Brachiaria, the duplication of sexual genotypes of *Brachiaria ruziziensis* (SWEENE et al.,1981; GOBBE et al., 1981) and more recently of *B. brizantha* was decisive. It allowed the production of genetic variation through crosses between sexual and apomictic plants with the same ploidy level, which was not possible before. The knowledge about the mechanisms of apomixis and the possibility of controlling this system will extend the possibilities of introducing variability in *Brachiaria* but maintaining the apomictic character which is desirable for its propagation.

Overview of biotechnological tools

Apomixis is a heritable trait that can be examined by molecular markers and by detecting gene or genes responsible for apomixis. Molecular markers are of interest because they can facilitate precocious and large scale detection of apomicts being very useful in analysis of hybrids that otherwise have to be characterized cytologically in a time consuming work. This technology also gives information that can be useful for isolation of genes. Molecular markers co-segregating or linked to apomixis in grasses have been identified and are being used as tools to study the long region responsible for apospory (revised in CARNEIRO et al., 2006). Markers linked to a trait of interest could also be used in cloning apomictic genes (revised in OZIAS-AKINS et al., 2007) but this strategy may not be easy.

Attempts to convert sexual plants into apomicts are in progress. In one side, strategies of mutagenesis to modify the female gametophyte development of sexual plants are being studied in model plants such as rice (KHUSH et al., 1994) and *Arabidopsis* (RAY, 1994; CHAUDHARY; PEACOCK, 1994; CHAUDHURY et al., 1997; VIELLE-CALZADA et al., 1998). Genes involved in the control of particular developmental stages that are characteristic of apomixis, have been identified (GROSSNIKLAUS, 2001; KOLTUNOW; GROSSNIKLAUS, 2003). Recently, a genotype of *Arabidopsis thaliana* denominated MiMe, in which meiosis is substituted by mitosis was created through a combination of mutations (D'ERFURTH et al., 2009). On the other side, transfer of apomixis to sexual plants can be envisaged either by crossing sexual plants with a wild apomictic relative or by genetic transformation of sexual plants with genes capable of inducing apomictic development. In fact, many efforts have been applied into introgression of apomixis into cultivars (SAVIDAN et al., 1994; OZIAS-AKINS et al., 1993, 1998; SAVIDAN et al., 1994, 1996; GRIMANELLI et al., 1996; SAVIDAN, 2001) but resulted in plants that cannot be used agronomically (SPILLANE et al., 2004).

Identification of apomictic related genes, its isolation and characterization with the main objective of using genetic transformation of sexual cultivars to introduce apomixis genes that can control this mechanism are been carried on. The isolation of mRNA transcripts from ovaries or flowers and the comparative gene expression profile in sexual and apomictic plants have been done for grasses such as *Pennisetum ciliare* (VIELLE-CALZADA et al., 1996), *Brachiaria* (LEBLANC et al. ,1997; DUSI, 2001; RODRIGUES et

al., 2003), *Paspalum notatum* (PESSINO et al., 2001), *Panicum maximum* (CHEN et al., 1999) *and Poa pratensis* (ALBERTINI et al., 2004; 2005).

Our group at Embrapa has been studying apomixis in *Brachiaria* for many years (CARNEIRO et al., 2007). We have characterized apomictic and sexual male and female sporogenesis and gametogenesis in sexual and apomictic accessions of *Brachiaria* and correlated them with visible or measurable phenotypes (DUSI; WILLEMSE, 1999a, 1999b; ARAUJO et al., 2000). In early stages of ovule development, near the archespore, the meiocyte or even after meiosis, near the megaspores, some somatic cells of the nucellus begin to differentiate. These cells have a characteristic of aposporous initials, previously described, that can be distinguished from the other nucellar cells by their large cell size, large nuclear size, dense cytoplasm, vacuolation and thick cell wall (NOGLER, 1984; KOLTUNOW, 1993; NAUMOVA; WILLEMSE, 1995). After vacuolation, one or more of these cells undergo directly through two mitotic divisions to form the four nucleated unreduced embryo sacs of the Panicum type. The nuclei are polarized in one side of the embryo sac, not always the micropylar side, and are easy to distinguish from the meiotic eight nucleated embryo sac of the Polygonum type. If the meiotic embryo sac development is not arrested a Polygonum type embryo sac can develop. In *Brachiaria* it is still not known whether the egg cell of this meiotic embryo sac can be fertilized giving rise to a progeny by means of sexual reproduction and therefore confirming the facultative nature of apomixis in *Brachiaria*.

In *B. brizantha,* mRNA was isolated from pistils or ovaries of sexual and apomictic plants and cDNA sequences related to sexual or apomictic embryo sac development were identified and are more detailed studies are under way. Some of the sequences showed homology with genes of known functions while others had their function predicted by means of elaborated bioinformatics analysis (RODRIGUES et al., 2003). In addition, many sequences had their temporal and spatial pattern of expression determined by *in situ* hybridization or real time PCR. Sequences with homology with MAP kinase, aquaporin, myosin were found to have distinguished expression in late stage of embryo sac development in special in synergids suggesting to to be related either to pollination or autonomous embryo development (ALVES et al., 2007).

To identify and clone genes related to the beginning of aposporic development as well as genes related to the egg cell activation, the expression analysis of the candidate genes has to be performed *in vivo*.

In order to understand the apomictic process in *Brachiaria*, we are working on the development of biotechnological tools (CARNEIRO; DUSI, 2002; 2004) that will in one side increase knowledge about the reproduction process and on the other side support further molecular work to study gene expression. Three biotechniques are under development and improvement.

1- In-vitro fertilization

In apomicts, viable reduced pollen grain formation often occurs normally in anthers (NOGLER, 1984a; CZAPIK, 1994) but a disorder during male meiosis is frequent even in plants that need fertilization of the polar nuclei in order to produce viable seeds (ASKER; JERLING, 1992). Meiotic abnomalies with consequent abortion of microspores or pollen grain, or a decrease in pollen grain fertility may occur (VALLE; SAVIDAN, 1996). In *B. decumbens* the apomictic access presents higher abortion during meiosis and also in mature pollen grains (DUSI; WILLEMSE, 1999).

The majority of the apomicts are facultative (NOGLER, 1984; ASKER; JERLING, 1992) i.e. they are able to generate at least part of its progeny by the sexual pathway. Nogler (1984) believes that all apomicts have the potentiality to reproduce by means of the sexual mechanism since there is always some residual sexuality even in the species where apomixis seems to be obligatory. Facultative apomixis, as represented by percentage of sexuality expressed at the embryological level, such as in *Brachiaria*, is not necessarily equivalent to the observations of off-types in the field (MILES; VALLE, 1991). Since in *Brachiaria* unreduced embryo sacs coexist side by side with the reduced ones, two questions are to be answered. 1- Is the reduced embryo sac egg cell able to be fertilized? 2- How does the unreduced egg cell avoid being fertilized?

Embryogenesis can occur before the arrival of the pollen tube (NOGLER, 1984), or there might be a physical barrier in unreduced egg cell wall that inhibits fertilization (SAVIDAN, 1989; ASKER; JERLING, 1992). Both ideas are supported only partially in species with diplospory or apospory. In many species embryo development depends on polar nuclei fertilization. The cell wall hypothesis could fit the system of some plants like *Pennisetum ciliare* (VIELLE et al., 1995). But the hypothesis could not be applied to all plants as in *Pennisetum* hybrids (CHAPMAN; BUSRI 1994), and *Panicum maximum* (NAUMOVA; WILLEMSE, 1995) where the apomictic mature embryo sac egg apparatus is comparable to the sexual one. It is possible that physiological or metabolic changes,

difficult to detect, may lead to changes in the egg apparatus structure and in early stages of embryo formation. As suggested by Vielle et al. (1995), some activation process may be taking place before any change in cell wall can be observed.

We have developed an *in vitro* pollination and fertilization method for *Brachiaria* spp. (DUSI et al., submitted). The presence of sperm nuclei inside an unreduced egg cell was observed indicating that the mechanism to prevent the unreduced egg cell fertilization is not likely to be a barrier that prevents the sperm nuclei to enter the cell. Although at this moment we do not have all the answers for the questions, *in vitro* fertilization seems to be a promising method not only to study the first events of fertilization but also to allow plant recovery through a 2n+n hybridization, which would indicate that a certain degree of variability among *Brachiaria* polyploid apomictics is possible.

2- Somatic Embryogenesis and genetic transformation

Optimized protocols for tissue culture are pre requisites to establish genetic transformation in *Brachiaria* (TOHME et al., 1996). *Brachiaria* is recalcitrant for both, plant regeneration and transformation. The desired homogeneity in somatic embryos resulted from tissue culture is very difficult to obtain Our group has been working on somatic embryogenesis using different tissues or organs of *B. brizantha* as explant sources (LENIS-MANZANO, 1998; SILVEIRA et al., 2003; CABRAL et al., 2006; 2008; OLIVEIRA et al., 2008). Among all the explants tested, the use of cell suspensions obtained from mature seed embryogenic calli is the most promising. A recent publication reported regeneration from *B. ruziziensis* (ISHIGAKI et al.,2009).

Gene introduction using either biolistic or *Agrobacterium*-mediated transformation is being optimized in cell suspension systems (CABRAL et al., 2008). These methods of gene introduction are being improved to overexpress and silence candidate genes. The silencing mechanism using RNA interference, RNAi, (WATERHOUSE; HELLIWELL, 2003; ZAMORE, 2004; WASSENEGGER, 2005; SCHWAB et al., 2006) allows the establishment of gene function through the introduction of a sequence of double strand RNA (dsRNA), hairpin RNA (hpRNA) or artificial microRNA (SCHWAB et al., 2006; OSSOWSKI et al., 2008) to induce the silencing of the target gene. It is currently being tested to silence three gene sequences, a miosin that might have a role in the fertilization of the egg cell and degeneration of synergids; a recombinase that is expressed during megasporogenesis in apomictic plants, and a mitogen activated protein kinase, MAPK that is probably

associated to the autonomous development of the embryo (ALVES et al., 2007). The same sequences are being over-expressed using the complete coding sequence under the control of strong monocot promoters, that were selected for their good performance in *Brachiaria* (SILVEIRA et al., 2003), Ubi1 from maize and Act1 from rice. In addition to *Brachiaria*, rice, a sexual plant which has orthologous genes of these sequences, is being used for silencing and overexpressing experiments due to its well established transformation protocol.

3- Androgenesis for haploid production

The importance of haploids in the study of apomixis lies mainly in the need fro uncovering the relationship of apomixis and poliploidy in the genus *Brachiaria*. For breeding, haploids and double haploids are important for the possibility of generating new genotypes that can be used as progenitors in intra specific crossings resulting in new hybrid populations. Haploid plants will also provide an important genetic background for genetic transformation experiments that can result in great improvements both for breeding and apomixis studies. Androgenesis is the embryo formation from microspores and is largely used to produce haploid and double-haploid plants. Three events characterize androgenesis (MARASCHIN et al., 2005): 1- acquisition of embryogenic potential by the microspore; 2- initiation of cell divisions; 3- pattern formation. Our group has been working on the development of haploids. We are characterizing the anther and microspore cultures using microscopic observations. Microspore divisions and embryo-like structures were obtained. Expression of genes related to embryogenesis will be used as markers that will allow following the process of acquisition of cell embryogenic competence.

The biotechnological tools that have been developed have already provided important information about aposporous apomixis in *Brachiaria* spp. and are being used towards a better understanding of the regulation of the apomixis reproductive mechanism. Our group aims to continue developing methodologies that will eventually lead to a more efficient breeding program in *Brachiaria*.

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