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Identification of ovule specific proteins associated with apomixis and sexuality in *Cenchrus ciliaris*

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Abstract
In order to identify possible specific markers associated with pre and post meiotic stages of megasporogenesis, ovaries representing pre and post-meiotic stages of apomictic and sexual segregants of an F_2 mapping population of *Cenchrus ciliaris* were studied using native and SDS-polyacrylamide gel electrophoresis. Four differentiating proteins of ovaries showed different patterns of associations. While one 97 kDa protein was associated with ovaries at pre-meiotic stage, one 80 kDa protein was absent in ovaries at post-meiotic stage. A 60 kDa protein was observed in pre and post meiotic sexual ovaries and a 50 kDa protein was associated with pre-meiotic ovaries of both sexual and apomictic plants. Dendrogram analysis using protein polymorphism indicated two groups, one with pre-meiotic apomictic and sexual ovaries while the other included post meiotic apomictic and sexual ovaries. Esterase enzyme exhibited maximum polymorphism among four different types of ovaries. Out of 43 putative alleles scored across 15 isozyme loci of six enzymes, six alleles of Est-1 were associated with pre meiotic ovaries of apomictic plants among which 3 were conspicuously absent in sexual plants altogether. Dendrogram based on isozymes indicated two groups, one indicating three stages *viz*., pre and post-meiotic sexual as well as post meiotic apomictic ovaries while pre-meiotic apomictic ovaries showed a distinct group.

Key words: Apomixis, Megasporogenesis, Isozymes, Protein polymorphism, Fodder grass.

Introduction

Apomixis is an asexual mode of reproduction through seeds. This trait is highly desirable for fixing heterosis in F_1 hybrids with significant implications for crop improvement (Dwivedi *et al*., 2007). Apomixis is prevalent in several plant species but predominant in families such as *Rosaceae, Compositae and Poaceae* (Richards, 1986, Ozias-Akins 2006). Engineering apomixis into cultivated crop species can revolutionize agriculture and confer commercial and social benefits for both industrialized and developing countries (Jefferson, 1994; Koltunow *et al.,* 1995; Grossniklaus *et al*., 1998, Ozias-Akins 2006).

Apomictic progenies will be genetically uniform and will be identical to the mother plant. Hence various biochemical and molecular techniques can be employed to distinguish apomictic and sexual progenies. There have been only few studies of biochemical characterization of the process of megasporogenesis and megagametogenesis (Gustine *et al*., 1996). In that study, 12 of the 22 isozymes showed polymorphism, but none co-segregated with apomixis and none among 308 spots of proteins obtained through 2-D polyacrylamide gel electrophoresis using pistils at meiotic and pre-meiotic stages co-segregated with reproductive mode. In an another study, Gounaris *et al*. (1991) analyzed proteins from pistils and stamens at sporogenesis and enlargement of the gametophyte prior to stigma exertion, anthesis or fertilization using 2-D electrophoresis where four stamen specific proteins were detected.

During this study, the objective was to find out protein and isozymes associated with pre-meiotic and post-meiotic stages of megasporogenesis and megagametogenesis during sexual and apomictic development that constitute an initial step towards a detailed cellular and molecular analysis of apomictic reproduction. Differential expression of proteins and isozymes could indicate the mode of regulatory systems working for induction of apomixis. This study will also help in further characterization of proteins associated with apomixis. This is the first ever study undertaken to compare protein and isozyme patterns across

pre-meiotic and post-meiotic megasporogenesis in *Cenchrus ciliaris*.

Materials and Methods

Plant material: A single sexual plant (IG-96-443) of *Cenchrus ciliaris* (Gupta *et al*., 2001) was open pollinated to obtain half-sibs which showed either obligate or facultative apomixis. Few facultative apomictic half-sibs were further selfed to get F_2 segregants that showed clear segregation for the mode of reproduction. One obligate sexual (7-4) and an obligate apomictic (7-18) plant were used for our study (Dwivedi *et al*., 2007). These plants were multiplied vegetatively to get large number of ovaries for extraction. Since the half-sib parent of F_2 segregants was a product of open pollination, no information regarding the paternal parent was available. Ovaries from these plants were fixed, cleared by methyl salicylate and examined under phase contrast microscope (Young *et al*., 1979) for differentiating pre-meiotic and post-meiotic stages during megasporogenesis and megagametogenesis.

***SDS-Polyacrylamide gel electrophoresis*:** Inflorescences were collected at the pre-meiotic stage (stigma 10% exerted) and post-meiotic stage (stigma 100% exerted) and were stored in an ice basket. Pistils were collected, stigma and style of pistils were removed. About 1000 ovaries were collected in 500ml of extraction buffer (50mM Tris-HCl, pH 6.8 containing 10% glycerol, 2% SDS, 5% b-mercaptoethanol, 0.001% bromophenol blue) in Eppendorf tubes and were stored at 4°C. Extracted Proteins were quantified by spectrophotometer and equal volume of protein was loaded in each well.

Ovules were thoroughly crushed using a glass rod and centrifuged at 10,000 rpm for 20 minutes at 4°C. The samples were heated at 96°C for 5 minutes and were immediately used for electrophoresis. Best resolution was achieved when the samples were run on 9% resolving and 4% stacking gel in a running buffer (Tris-Glycine buffer, pH 8.3) (Laemmli, 1970) at 70 volts. After the tracking dye reached the bottom of the gel, the slabs were removed and stained in staining solution (0.25g Coomassie brilliant blue R-250, 45 ml methanol, 10 ml acetic acid and 45 ml distilled water).

***Isozyme analysis*:** Sequential extraction and subsequent analysis of ovular enzymes by native polyacrylamide gel electrophoresis was carried out as per procedure described by Soltis and Soltis (1989).

Inflorescences from F_2 plants of *Cenchrus* were collected in ice at the pre-meiotic (stigma 10% exerted) and post-meiotic stages (stigma 100% exerted). Ovaries of both the plants were isolated in extraction buffer (100mM Tris-HCl, pH 6.8 with 10% sucrose, 1% PVP40000 and 10mM β-mercaptoethanol (Soltis and Soltis, 1989) and stored at 4°C. They were thoroughly crushed in 500µl of cold extraction buffer in Eppendorf tubes using a glass rod and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was frozen at –20°C until used for electrophoresis.

Electrophoresis was carried out in a vertical discontinuous polyacrylamide gel system (stacking 4%, resolving 7.5%) as per Laemmli buffer system (1970). Isozymes of Esterase, G6PDH, Acid phosphatase, Phosphoglucomutase, Phosphoglucoisomerase, Leucine amino peptidase, Hexokinase, Aspartate amino transferase and Super oxide dismutase were studied. The gels were stained as described by Soltis and Soltis (1989).

Based on zone of activity of each enzyme, putative loci were assigned. Within each locus, the bands were numbered starting from slowest band to fast migrating band. Bands were scored as either present (1) or absent (0) and a Jaccards similarity index was derived. Phylogenetic tree was constructed using UPGMA method (Sneath and Sokal, 1973). Each band was considered as a putative allele in this study.

Results and Discussion

Protein polymorphism

Native and SDS-PAGE analysis: A total of 17 protein bands were observed out of which four exhibited polymorphism among ovaries of sexual and apomictic plants (Table 1). A 97 kDa protein was associated with only pre meiotic sexual ovaries while a 80 kDa protein complement was absent only in apomictic post meiotic ovaries. A 60 kDa protein was observed in sexual ovaries undergoing both pre-meiotic and post-meiotic stages, and it was conspicuously absent in both the stages of apomictic ovaries. Another 50 kDa protein was associated with pre meiotic ovaries of both the sexual and apomictic plants, while it was absent in post meiotic ovules of both the plants (Fig 1).

Dendrogram analysis based on protein banding pattern indicated two major groups. One group included pre-meiotic apomictic and sexual ovaries, while the post-meiotic and sexual ovaries grouped into another group (Fig. 2).

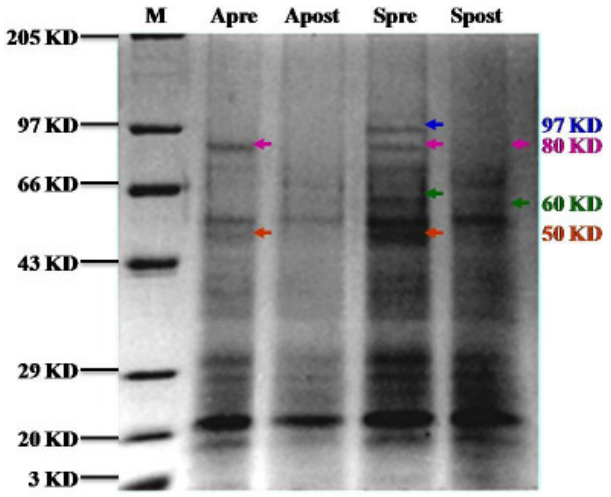

Fig 1. Protein polymorphism among pre and post meiotic ovules of apomictic (Apre & Apost) and sexual (Spre & Spost) F_2 segregants of Buffel grass, M (molecular weight marker).

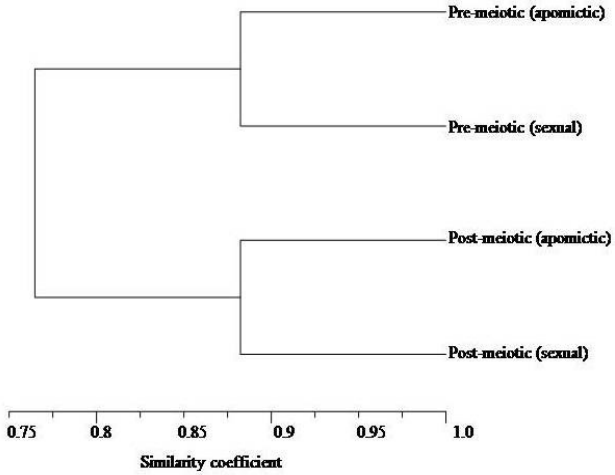

Fig 2. UPGMA dendrogram based on protein polymorphism analysis depicting relationship among pre and post meiotic ovules of apomictic and sexual plants.

Our results clearly showed that stage specific proteins are expressed during apomictic and sexual developmental pathways. The earlier studies on this grass could not separate any steady state proteins of pistils at either pre meiotic or post meiotic stages, although 308 spots showing nearly 12% polymorphism have been observed (Gustine *et al*., 1996), but none of the proteins were found to co-segregate with the reproductive mode. Whereas, Gounaris *et al*. (1991) had earlier reported 4 stamen specific proteins out of 308 spots examined. Our study also indicated that there might be specific genes regulating these processes that have their protein products, which are detectable.

Stage specific proteins have also been detected in the reproductive tissues of the early stages of formation of sporogenous tissues (Kahlem, 1976) or the microspores (Singh *et al*., 1985). Proteins that are both organ-specific and stage-specific are interesting because their correlation with form and function indicated possible role for the proteins. All the four proteins in our study differentiated specifically with either pre or post-meiotic apomictic or sexual ovules. Results of study by Vielle *et al*. (1995) also corroborated our findings clearly indicating differences between the early fertilization events in sexual and apomictic female gametophytes of *Cenchrus ciliaris*. They compared sexual and aposporous egg apparatus (synergids and egg cell) of both sexual and apomictic F_1 segregants unpollinated and pollinated pistils prior to the pollen tube entry into the female gametophyte.

In Kentucky blue grass using SDS-PAGE of endosperm storage proteins, Agafonov *et al*., (2004) reported that the endosperm development as a result of inbreeding or apomixis may be identified by the combination of protein markers of the maternal and pollinator genotypes in the endosperm.

Sequencing and expression analysis of n20gap-1, a lorelei-like gene from *Paspalum notatum*, which encodes an apomixis associated GPI-anchored protein (Felitti *et al*., 2011) indicated that lorelei-family genes present a minor activity peak at pre-meiosis and a major one at anthesis. The apomictic genotype analyzed showed a differential activity at pre-meiosis, post-meiosis and anthesis with respect to a sexual genotype. *In situ* hybridization analysis revealed expression of n20gap-1 in integuments, nucellus and the egg-cell apparatus. This supports our observation that proteins are differentially expressed at different growth stages during apomictic and sexual development.

Isozyme polymorphism: Extracts from four different kinds of ovaries of obligate apomictic and sexual plants were analyzed for six different enzyme systems that were polymorphic. The enzyme systems of Phospho-glucoisomerase, Leucine amino peptidase and Hexokinase did not show polymorphism among different stages. Enzymes exhibited polymorphism among ovaries at 4 stages of development. Out of 43 putative alleles scored among 15 loci, pre-meiotic stage ovaries exhibited higher number of alleles (37), while post-meiotic stage ovaries recorded the least (24) (Table 2). Maximum polymorphism was observed for esterase

enzyme followed by phospho-glucomutase. All the 6 alleles of Est-1 were associated with pre-meiotic stage apomictic ovaries but were absent during pre-meiotic sexual ovaries. Three alleles were conspicuously absent in ovaries of sexual plants altogether. Thus expression of isozymes could differentiate pre-meiotic sexual from apomictic types. Of the 14 loci, three loci *viz*. ACP-1, ACP-2 and AAT-2 did not reveal polymorphism across the four stages. The zymogram pattern is shown in Fig. 3.

Dendrogram analysis based on similarity of isozymes indicated grouping of pre and post meiotic sexual types and post meiotic apomictic types was distinct (Fig. 4). This is in contrast to dendrogram based on protein analysis.

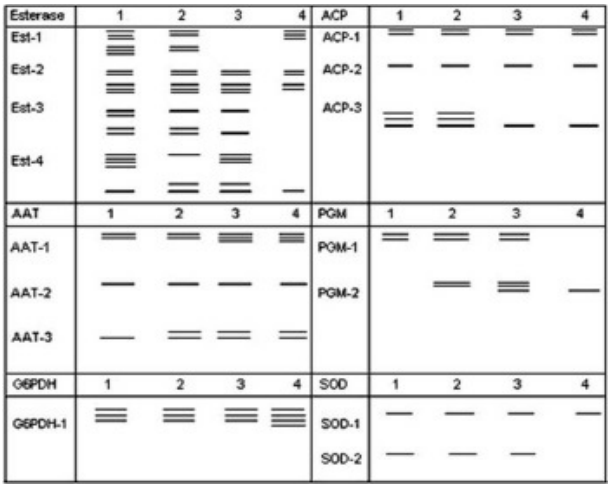

Fig. 3. Zymogram of six enzymes Esterase(Est), Acid phosphatase (ACP), Aspartate aminotransferase (AAT), Phosphoglucomutase (PGM), Glucose-6-phosphatase dehydrogenase (G6PDH) and Superoxide dismutase (SOD) showing polymorphism among pre and post meiotic apomictic and sexual ovules of F_2 segrgants *viz*.1.premeiotic apomictic 2.postmeioctic apomictic 3.premeiotic sexual 4.postmeiotic sexual.

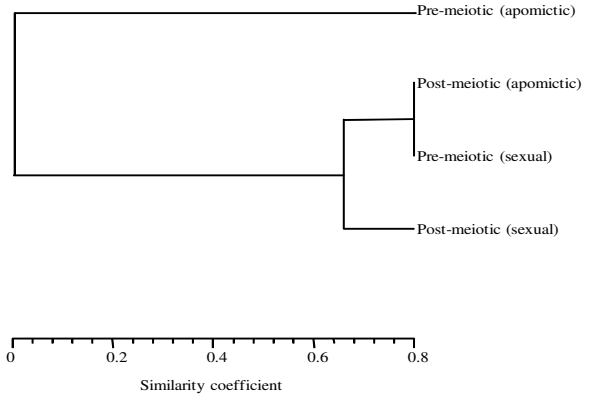

Fig. 4: UPGMA dendrogram based on isozyme polymorphism analysis depicting relationship among pre and post meiotic ovules of apomictic and sexual plants

Analysis of isozyme banding pattern of six enzymes namely Esterase, Acid phosphatase, Aspartate amino transferase, Phosphoglucomutase, Glucose-6-phosphate dehydrogenase and Superoxide dismutase has revealed polymorphism among ovaries of sexual and apomictic F_2 segregants. There was polymorphism between pre-meiotic and post-meiotic stages of both the types. Out of 43 putative alleles scored among 15 loci, 37 alleles were recorded in the pre-meiotic stages while only 24 alleles were recorded for post-meiotic stage. Maximum polymorphism of Esterase isozyme with all the 6 alleles of Est-1 associated with pre-meiotic stages clearly indicated their stage specific expression. 3 alleles of Est-1 were conspicuously absent in the ovaries of sexual plant, but 3 loci out of 15 loci namely ACP-1, ACP-2 and AAT-2 did not show polymorphism. This is the first report on differentiating isozymes during pre and post-meiotic gametogenesis.

Maximum polymorphism was observed for Esterase and Phosphoglucomutase, while Glucose-6 phosphate dehydrogenase and Superoxide Dismutase did not show significant polymorphism. This could be due to the type of material used, as they were genetically more homogenous. Moreover the genes encoding these enzymes may be conserved. On the contrary, Gustine *et al*., (1996) observed polymorphism in 12 out of 22 isozyme systems tested using leaf extracts where none of the isozyme co-segregated with apomixis. Higher polymorphism in their study could be due to the use of half-sib families, segregating for the mode of reproduction. Although half-sib families showed more polymorphism, the probability of identifying a co-segregating marker for apomixis is higher when F_2 segregants for the mode of reproduction are used.

In another study, isozyme polymorphism of the sexual pool was higher (69%) compared to apomictic pool (Assienan and Noirot 1995). Overall polymorphism was considerable indicating that apomixis does not lead to a reduction in diversity. The existence of three to four alleles at many loci (EST and ACP) leads us to assume *a priori* the presence of a high level of heterozygosity in apomicts by the maintenance of quadruplex or triplex structures. Assienan and Noirot (1995) did not observe any quadruplex type. This situation clearly showed that the advantage of the polyploidy-apomixis relationship is to the maintenance of within individual heterozygosity level of apomicts, which is higher than that of a diploid. In our study, ACP-3, Est-3 and Est-4 indicated specific alleles as sociated with apomictic ovaries, whereas AAT-1,

AAT-2, AAT-3 and G6PDH-1 loci exhibited alleles specific to sexual ovaries. In a survey of 50 enzymes of *Hordeum vulgare,* Pedersen *et al*. (1987) noted six anther specific enzymes. Koul and Bhargava (1986) also reported a few anther specific isoperoxidases during sporogenesis. Alcohol dehydrogenase activity first appeared after tetrads began to break apart in *Zea mays* (Stinson and Mascarenhas, 1985). None of the studies on isozymes so far was related to developmental stages of ovules.

The isozyme markers associated with apomixis which are identified in this study, if co-located within the Apo locus in the larger F_2 mapping population of *Cenchrus ciliaris* (Yadav *et al*., 2012), could be used for fine mapping, as isozyme markers follow Mendelian pattern of inheritance and are co-dominant in nature.

Table 1. Protein complements of different sizes associated with pre and post meiotic stages of apomictic and sexual F_2 segregants.

Protein complements	MW (kDa)	Apomictic (7-18)		Sexual (7-4)	
		Pre-meiotic	Post-meiotic	Pre-meiotic	Post-meiotic
1	97	-	-	+	-
2	80	+	-	+	+
3	60	-	-	+	+
4	50	+	-	+	-

Table 2. Isozyme polymorphism associated with pre and post meiotic stages of apomictic and sexual F_2 segregants

Enzyme	EC number	No. of loci recorded	No. of alleles			
			Apomictic		Sexual	
A. Isozymes			**Pre**	**Post**	**Pre**	**Post**
Esterase	E.C.3.1.1	4	20	15	12	8
Acid phosphatase	E.C.3.1.1.2	3	6	6	4	4
Aspartate aminotransferase	E.C.2.6.1.1	3	4	5	6	6
Phosphoglucomutase	E.C.5.4.2.2	2	2	4	5	1
Glucose 6 phosphodehydrogenase	E.C.1.1.1.4.2	1	3	3	3	4
Superoxide dismutase	E.C.1.15.1.1	2	2	2	2	1
Total		15	37	35	32	24
B. Protein Complements			15	12	17	14
Grand Total			52	27	49	38

Conclusions

The dendrogram analysis based on isozymes indicated grouping of pre and post meiotic sexual types including post-meiotic apomictic types delineating pre-meiotic apomictic type. This clearly implied the possibility of specific isozyme associated with pre-meiotic apomictic ovaries and seemed to play a definite role during early megasporogenesis than during the later stages of megasporogenesis and megagametogenesis of apomicts. Lack of isozyme polymorphism between pre and post-meiotic sexual ovaries indicated that the isozymes tested are not differentially regulated during these stages. Distinct grouping of isozymes of pre-meiotic apomictic ovaries was due to associated isozyme loci implying a different pattern of gene regulation during pre-meiotic megasporogenesis. These results will help further to characterize stage-specific proteins and isozymes to relate them to these developmental stages in reference to understanding expression of apomictic reproduction in *Cenchrus ciliaris.*

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