Generating higher ploidies (7*x* and 11*x*) in guinea grass (*Panicum maximum* Jacq.) utilizing reproductive diversity and uncoupled apomixis components

Guinea grass (*Panicum maximum* Jacq.), a high-yielding, perennial, multicut forage grass, is widely grown in tropical and subtropical countries. Tetraploid cytotypes ($2n = 4x = 32$) with obligate apomictic mode of reproduction are predominant in this crop, though plants with variant ploidies and sexual mode of reproduction have been rarely reported[1–3]. These features make guinea grass a model crop for polyploidy and apomixis research. Apomixis, a mode of clonal reproduction through seeds is widespread among grasses[4]. Studies are underway to harness 'genes' for apomixis for their potential use in the fixation of hybrid vigour[5]. Apomixis phenotype is generated when an unreduced egg cell (developed through apomeiosis in the ovule) develops without fertilization (i.e. parthenogenesis) followed with proliferation of the central cell to form endosperm (i.e. functional endosperm development). All these three components must be functionally linked to generate apomictic phenotype. Contrary to previous reports on one gene model of apomixis regulation, we have demonstrated independent regulation and recombination between components of apomixis[6]. In guinea grass, a total of eight pathways of seed development arising from recombination between the three apomixis components (apomeiosis, parthenogenesis and functional endosperm development) were identified[6]. Uncoupled (partitioned) apomixis components may generate higher frequencies of triploids (through fertilization of an unreduced egg cell) and haploids (parthenogenetic development of a reduced egg cell). We have utilized these recombination events to generate a ploidy series represented by 3*x*, 4*x*, 5*x*, 6*x*, 8*x* and 9*x* cytotypes from a single 4*x* progenitor following a hybridization-supplemented apomixis-components partitioning approach (HAPA)[3]. HAPA relies on utilization of uncoupled apomixis components and their appropriate supplementation with pollination strategy to manipulate the ploidy level of the progeny without using any external agent (such as chemicals).

In the present study, we report generation of novel ploidies and further enrichment of this series with 7*x* ($2n = 56$) and 11*x* ($2n = 88$) cytotypes. Till now, these plants represent the most exhaustive ploidy series in a crop plant.

Parents to generate 7*x* and 11*x* plants were selected from the ploidy series developed through HAPA[3]. A facultative aposporous 8*x* plant (identity GGPS 8*x* – 1, $2n = 64$) was used as maternal parent, whereas another 6*x* plant (identity 3/29/2, $2n = 48$) was used as pollinator (Figure 1). A crossing block scheme[3] was followed to facilitate cross-pollination. Mature seeds were hand-picked from female parent at regular intervals. Seeds were germinated in a nursery in the subsequent season and screened utilizing flow cytometric ploidy analysis from the leaf tissues for identification of any non-maternal ploidies. Plants representing novel ploidies were established separately and subjected to cytological and reproductive characterization.

Ploidy level of plants was estimated through flow cytometric measurement of DNA content of leaf tissues[3]. Young and disease-free leaves (2–3 cm^2) were chopped with a sharp razor blade in 2 ml extraction buffer (pH 7.0) containing 100 mM Tris-HCl, 5 mM $MgCl_2$, 85 mM NaCl, 0.1% Triton X100 and 1 μg/ml DAPI (4′-6-diamidino-2 phenylindole), for isolation of nuclei. The suspension was filtered through 30 μm mesh-width nylon filters and incubated for 10–15 min before measurement in a flow cytometer (Ploidy Analyzer PA I, Partec, Germany) equipped with the CA3 2.14/2004 software. Each sample was analysed in three replications and relative DNA content was estimated by proportion of the respective peaks (G1 phase cells) of the sample and the internal standard in a flow cytometer (FCM) histogram[7]. In the present study, 2C value corresponds to sporophytic DNA content. A tetraploid guinea grass cultivar, viz. Riversdale (1.77 pg per 2C DNA) as well as another grass *Pennisetum squamulatum* (7.26 pg per 2C DNA)[8], were used as internal standards in flow cytometric measurements.

Chromosome number was estimated from squashed anther preparations at the appropriate stage of division stained in 2% acetocarmine. Data on chromosome number and pairing were recorded in 25–50 PMCs at diakinesis and metaphase I stage of meiosis. Pollen fertility was estimated by proportion of well-stained and filled grains stained in glycerine/acetocarmine (1 : 1) from a minimum of 15 microscopic fields.

Mode of reproduction was estimated by methyl salicylate-mediated ovule-clearing whole-mount observed under differential interference contrast (DIC) microscope[9]. Ovules containing eight-nucleated embryo sacs (ES; two synergids, one egg cell, two polars and three antipodals) were considered sexual (*Polygonum*-type), while four-nucleated ES (two synergids, one polar nucleus and one egg cell, antipodals absent) were considered as *Panicum*-type aposporous[10]. When both types of ES were observed in ovules of the same plant, it was termed as facultative aposporous/sexual (hereafter called facultative). The proportion of sexual and aposporous ES was also recorded.

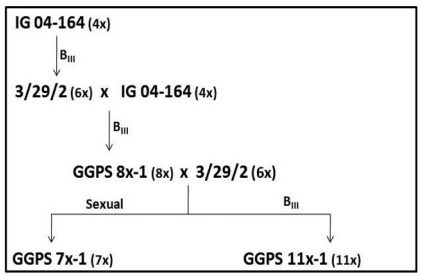

Figure 1. Origin and pedigree of the plant material. Pathway of fertilization events is shown as B_{III} ($2n + n$; unreduced female gamete fertilized with reduced male gamete) or sexual ($n + n$; fertilization of both reduced gametes). Ploidy level of respective plants is shown in parenthesis.

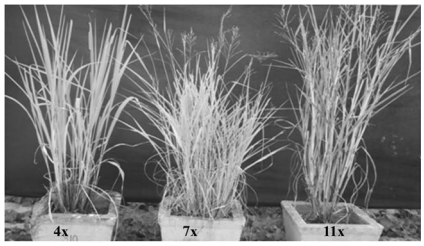

Figure 2. Overall plant morphology of 7*x* and 11*x* compared to 4*x* variety.

Table 1. Cytological and reproductive characteristics of 7*x* and 11*x* plants along with the 8*x* maternal parent

Trait/characteristic	GGPS 8*x* – 1	GGPS 7*x* – 1	GGPS 11*x* – 1
Chromosome number (2*n*)	64	56	88
Average chromosome configuration	$2.2_{I} + 13.5_{II} + 1.6_{III} + 4_{IV} + 1.3_{V} + 1.2_{VI} + 0.05_{VII}$	$2_{I} + 13.4_{II} + 2.6_{III} + 3.2_{IV} + 0.6_{V} + 0.6_{VI}$	$3.75_{I} + 15.25_{II} + 3.75_{III} + 4.75_{IV} + 2.25_{V} + 1.75_{VI} + 0.25_{VII}$
2*n* DNA content (pg)	3.6	3.2	5.0
Pollen fertility (%)	95	68	57
Embryo sac (ES)	65% aposporous 35% sexual 55% multiple	72% aposporous 28% sexual 38% multiple	100% aposporous 72% multiple

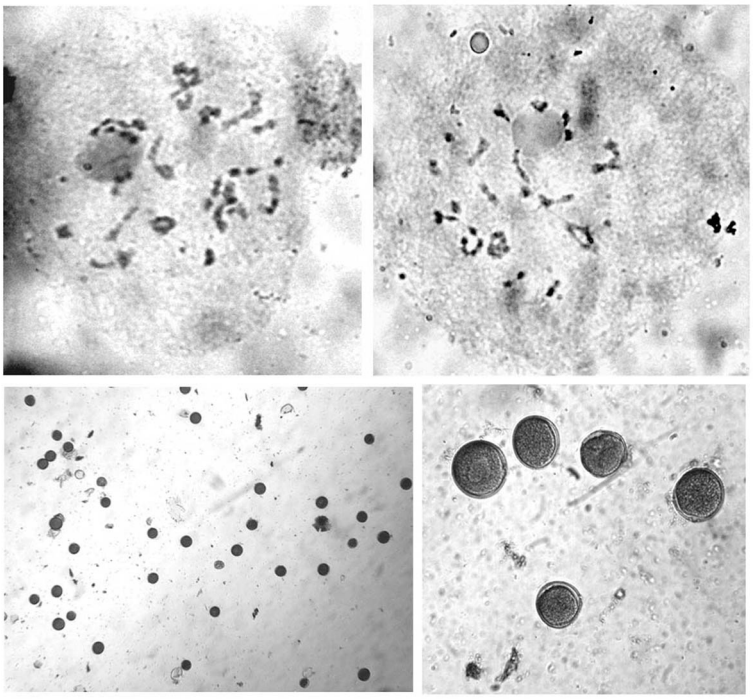

Figure 3. (Upper panel) Meiosis (diakinesis) and (lower panel) pollen fertility in 7*x* plant.

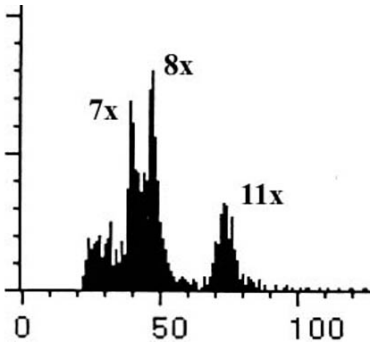

Figure 4. Flow cytometric measurement of relative DNA contents. Respective peaks are superscripted with the ploidy levels. *X*-axis, Photo-illumination; *Y*-axis, Number of cells.

The maternal parent GGPS 8*x* – 1 was characterized as facultative having 65% aposporous ES and 55% multiple ES, as revealed by ovule clearing, and set seeds upon open pollination. A total of 209 plants germinated from open-pollinated seeds collected from GGPS 8*x* – 1. Flow cytomteric measurement of ploidy from leaves of these seedlings revealed 205 plants with 8*x* ploidy (maternal types); however, four plants with odd ploidies (non-maternal types); represented by 7*x* (one plant), 11*x* (two plants) and 12*x* (one plant) ploidy levels were also recovered. These four plants were transferred to pots for better establishment. While one 11*x* and the 12*x* plant could not survive, two plants representing 7*x* and 11*x* established well, and were designated with identity GGPS 7*x* – 1 and GGPS 11*x* – 1 respectively (Figure 2). These plants were subjected to detailed characterization, including growth habit, cytology and reproductive behaviour, compared to their 8*x* parent (Table 1). Both the plants GGPS 7*x* – 1 and GGPS 11*x* – 1, as well as maternal GGPS 8*x* – 1 were perennial, multitillered, multicut with high regeneration potential typical of guinea grass cultivars.

GGPS 7*x* – 1 contained 2*n* = 56 chromosomes (Table 1), showing $2_{I} + 13.4_{II} + 2.6_{III} + 3.2_{IV} + 0.6_{V} + 0.6_{VI}$ as an average chromosome configuration (Figure 3). The plant was male fertile with 68% pollen stainability, though variable sized pollen grains were of common occurrence (Figure 3). This plant showed a 2C DNA content of 3.2 pg (Figure 4), that was proportional to its ploidy. It was facultative aposporous in the mode of reproduction, exhibiting 72% aposporous ES (four-nucleated *Panicum* type; Figure 5). Multiple ES were seen in 38% ovules of this plant. Well-filled seeds could be obtained upon self-pollination of this plant.

The plant GGPS 11*x* – 1 had 2*n* = 88 chromosomes (Figure 6), with $3.75_{I} + 15.25_{II} + 3.75_{III} + 4.75_{IV} + 2.25_{V} + 1.75_{VI} + 0.25_{VII}$ as average meiotic chromosome configuration, and a 2C DNA content of 5 pg (Figure 4) matching with its ploidy level. The plant was semi-sterile, showing 57% pollen stainability and high variability in pollen size (Figure 6). It was obligate aposporous with high frequency of occurrence of multiple ES (72%; Figure 5). In general, the plant

exhibited larger size of gynoecium and ovary, and characterized with occurrence of trifid stigma (70% expressivity), a feature rarely reported in guinea grass (Figure 7). This plant was a shy seed-setter upon self-pollination.

In general, these plants with higher ploidies (7*x*, 8*x* and 11*x*) exhibit characters typical of artificially synthesized higher polyploids[11]. It includes their slower growth compared to the predominant 4*x* cytotypes, multivalent formation in meiosis, variable sized pollen grains, semi-sterility and reduced seed set.

Plants representing four ploidy levels, viz. 7*x*, 8*x*, 11*x* and 12*x* were obtained from the crosses between 8*x* and 6*x* parents. Origin of 8*x* progeny, which was in fact represented by majority of the progenies, would have originated from self-pollination of 8*x* plants. The maternal parent (GGPS 8*x* – 1) was a facultative aposporous plant with presence of up to 35% of sexual embryo sacs. Origin of 7*x* plant is explained on the basis of fertilization between both reduced female and male gametes (Figure 1). Egg cell in sexual ES of facultative aposporous GGPS 8*x* – 1 is expected to be 4*x*, which when fertilized with male gamete (3*x*) from the 6*x* parent generated a 7*x* progeny through *n* + *n* hybridization (B_{II}) pathway. The 11*x* plant would have a B_{III} origin, whereby the unreduced egg cell (8*x*) from an aposporous ES of GGPS 8*x* – 1 fertilized with reduced (3*x*) male gamete from the 6*x* parent. Formation of these plants represented sexual pathway (reduced egg cell–zygotic embryo–pseudogamous endosperm) and B_{III} pathway (aposporous egg cell–zygotic embryo–pseudogamous endosperm) respectively, as identified in guinea grass seed development[6]. Similarly, the putative 12*x* progeny, even though the plant failed to survive, would possibly be a B_{III} hybrid originating from self-pollination, i.e. 8*x* + 4*x*.

Both the plants (7*x* and 11*x*) showed apomictic mode of reproduction, though

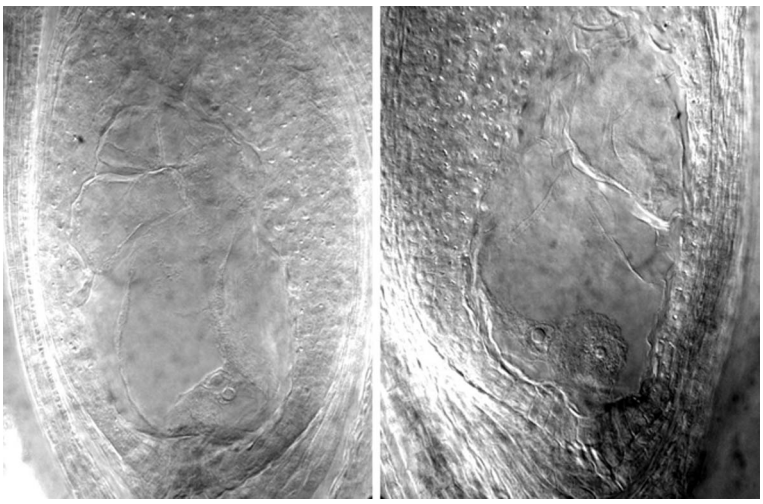

Figure 5. Representations of a sexual ES in 7*x* (note the presence of antipodals) and two aposporous ES in 11*x* plant.

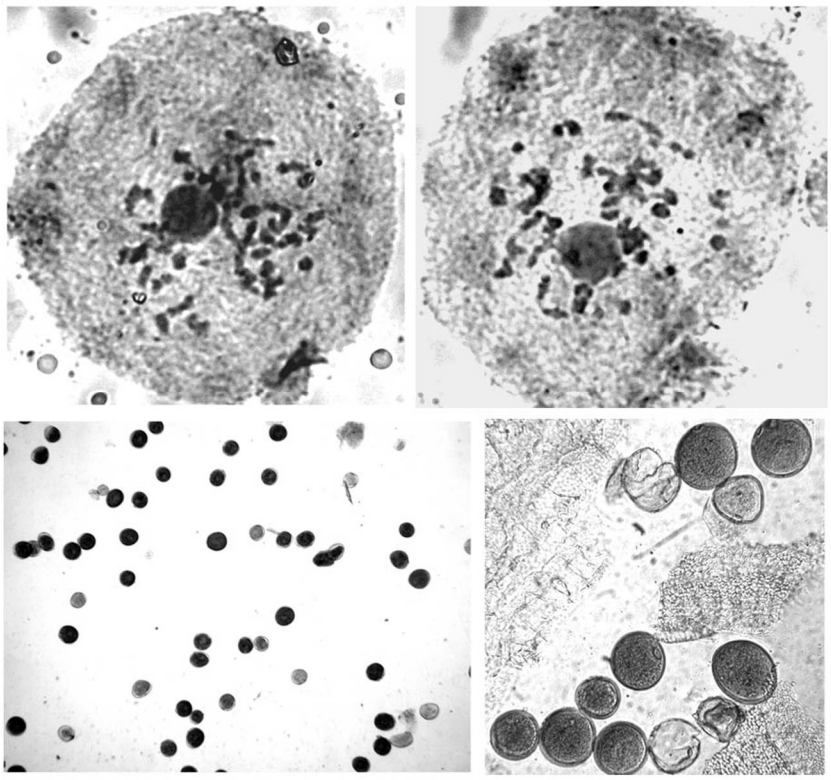

Figure 6. (Upper panel) Meiosis (diakinesis) and (lower panel) pollen fertility in 11*x* plant.

Figure 7. Dissected gynoecium showing trifid stigma in 11*x* plant.

7*x* was facultative while the 11*x* was an obligate aposporous. Their parents (GGPS 8*x* – 1 and 3/29/2) were also facultative and thus believed to carry genetic information for both apomictic as well as sexual modes of reproduction. Segregation of genes determining mode of reproduction in progenies may be the cause of this variation. Apomixis and its components are under genetic control[4]. It may be noted that all the pedigree discussed here (6*x*, 8*x*, 7*x* and 11*x*) had a common 4*x* ancestor (IG 04-164). This plant had very high B_{III} forming capacity which was utilized to generate 6*x* (3/29/2) and 8*x* (GGPS 8*x* – 1) cytotypes[3]. In fact, GGPS 7*x* – 1 and GGPS 11*x* – 1 represent backcross derivatives of IG 04-164. It may be observed that high B_{III} forming capacity from IG 04-164 was inherited to subsequent generations, i.e. 3/29/2 and GGPS 8*x* – 1 (refs 3 and 6). Formation of B_{III} hybrids is explained by uncoupling events between the two apomixis components, viz. apomeiosis and parthenogenesis. This resulted in loss of parthenogenetic capacity of the egg cell, and hence required fertilization of the (unreduced) egg cell for development (zygotic embryogenesis). Such hybrids with 3*n* constitution are resources for adding monoploid genomes[12], manipulating ploidies[13] as well as identification of genes modulating in response to change in ploidy and mode of reproduction[5,8,14].

Guinea grass is a model system for both apomixis and polyploidy research. It has shown a high flexibility to tolerate a great range of ploidies (3*x* to 11*x*) and chromosome numbers (2*n* = 24 to 88)[3,15,16]. Being perennial and vegetatively propagated, all these plants representing various ploidies can be easily maintained and simultaneously analysed. Similarly, this crop has demonstrated 'elasticity' in tolerating modifications in typical seed development patterns, especially the deviations from two maternal: one paternal genome ratios in the endosperm. Suitably enriched ploidy series, as reported in the present study in guinea grass, offers a system to understand this mechanism, especially in view of ploidy-dependent modifications in traits as well as evolution of polyploids. Nonetheless, it would be more interesting to generate higher ploidies following the same approach.

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Artificial pollination of Dove Orchid (*Peristeria elata* Hook.) in India for multiplication

The Neotropical orchid genus *Peristeria* Hook. includes seven species distributed from Costa Rica in Central America to the Amazonia, lowlands of Bolivia and Brazil (South America)[1]. Among these is *Peristeria elata* Hook., better known as Dove Orchid or Holy Ghost orchid, a native of Costa Rica, Panama, Venezuela and Colombia[2]. For over a century this species has been under cultivation in Kerala, southern India for its strikingly beautiful dove-shaped flowers which are also uniquely fragrant. Kumaranasan (1873–1924), one of the modern poets and social reformers of Kerala wrote a poem on this orchid which was published in the Malayalam magazine *Athmaposhini* in 1916. What enchanted the poet was the little dove look-alike, formed by the column and the lip, inside the flower and the ethereal fragrance. He called it '*Kapothapushpam*' ('*Kapotham*' in Malayalam means a dove and 'pushpam', a flower) reminiscent of the dove-shaped interior of the flower.

In its home country *P. elata* Hook. is called Dove Orchid due to the apparition of a dove formed by the beaked anther, column and the lip, all of which together constitute a dove in flight. The resemblance is so striking that the natives of Panama and the Spaniards call this 'El Spirito Sancto', or flower of the 'Holy Ghost'[3]. The plants are found at low to medium elevation under shaded conditions on the margins of grasslands and on rocky outcrops in the forest[4]. Though once common in the native country, this species has now become rare obviously due to over collection[5].

When this species is in bloom in South India, especially in the garden of our