Proc. Indian natn. Sci. Acad. **B53** No. 3 pp. 253–258 (1987)

Initiation of Androgenesis in Rice (*Oryza sativa* L. var Taipei 309)

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(Received 9 February 1987)

Initiation of androgenesis was studied in the uninucleate and binucleate pollen grains of rice variety Taipei 309 in liquid medium N 6 Grains showed trimorphism in their response to culture prior to initiation of division, i.e. highly enlarged empty grains, medium enlarged responsive grains and unenlarged unresponsive grains. Three pathways of androgenesis were observed. Pathway I, where initiation occurred in the uninucleate pollen grain, was the most frequent. Pathways II and III occurred in binucleate pollen which had already differentiated into vegetative and generative nuclei. In Pathway II, only the vegetative nucleus took part in the formation of the pollen callus while in Pathway III, generative nucleus divided first followed by the division of the vegetative nucleus and both contributed to the development of the callus. Wall formation occurred after 16 or more free nuclei were formed. Callus was released by the gradual disintegration of the exine over a period of 6 to 8 days. Direct formation of embryos from pollen was not observed. Early fragmentation of calli and their independent development indicated that all calli recovered after 35-40 days from culture plates may not be arising from individual pollen grains. It also suggested the possibility of distortion of progeny ratios in linkage and gene mapping studies based on anther derived plants. Polyploidization of haploid cells was observed very early in the development of the callus.

Key Words: *Oryza sativa*, **Androgenesis, Pathways, Callus release, Fragmentation, Polyploidization**

Introduction

Although considerable progress has been made in anther culture of rice, the response is generally low and genotype-specific. The lack of an efficient and dependable technique for the production of haploids and homozygous diploids from them in large numbers has severely limited the use of this tool for crop improvement and genetic studies. Moreover, knowledge of the basic aspects of the mechanism of induction of androgenesis still remains insufficient.

Early microspore divisions in rice during androgenic embryoid initiation have been reported by Guha et al. (1970). Uninucleate microspore was observed to undergo mitosis in culture to give rise to two morphologically alike cells which together produced multicellular pollen grains (Guha-Mukherjee 1973, Raina & Iyer 1974). It was also observed that during initial stages of development, pollen embryoids got attached to the wall of the anther by a suspensor-like outgrowth (Guha-Mukherjee 1973). Later on, first division of the uninucleate microspore was reported by Chen (1977) to be asymmetrical to form a large vegetative nucleus and small generative nucleus of which the vegetative nucleus formed the callus while the generative nucleus degenerated. The various types of segmentations in microspores of rice anthers cultured up to 12 days have been described (Chen & Wu 1983) and the pathways of androgenesis have been reported by Qu and Chen (1984). Here we report our observations on the initiation of androgenesis in both uni- and binucleate microspores upto the formation of callus and more specifically the release of callus from the exine, its fragmentation and polyploidization, with a

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view to adding more fundamental information about the process of androgenesis.

Materials and Methods

A *japonica* rice variety, Taipei 309, was used for culture. Panicles of 5–10 cm auricle distances were excised from the first 4 to 5 tillers from each plant, surface-sterilized in 20% (w/v) commercial chlorox and subjected to a cold shock of 8°C for 8 days. Pollen development stage was determined by fixing 1–2 spikelets from the top and bottom halves of each boot in acetic-ethanol (1:3) with ferric chloride (0.5%) as mordant for 24 hr and then squashing the anthers in 2% acetocarmine. Boots with spikelets in mid- or late-uninucleate stage and early or mid-binucleate stage were used for inoculation. N6 liquid medium (Chu 1978) with 6% sucrose and 2.0 mg 2,4-D/l was used. Between 50 and 60 anthers were plated per six ml of medium in 60 mm × 15 mm plastic (1007-Falcon) petri plates. Single anthers were cultured in microwell plates (Linbro 76003-05) with 0.2 ml medium per well. Plates were incubated in dim light at 25°–28°C. Samples of anthers were collected at one day-intervals for 35 days after plating and fixed as above for cytological studies. Observations were made at one day-interval on living pollen grains released into the medium by the anthers and undergoing division in the petri plates and microwells, and on pollen grains from the fixed anthers which were smeared in 2% acetocarmine.

Results

Anthesis occurred within 24 to 48 hr after plating and those that did not respond up to 6 days generally did not dehisce later. The unresponsive anthers usually turned brown and appeared dried while anthers with developing grains appeared pale, healthy and swollen. Observations made on anthers cultured singly on microwell plates showed that from 16 up to 432 grains were released into the medium from one anther within 6 days of plating. Some grains still remained within the anther walls even in the fully dehisced anthers. It was possible to observe three categories of pollen after 24 hr of culture on the basis of morphological and physiological changes.

(i) Highly enlarged (up to two to three times their initial size) pollen which appeared empty or highly vacuolated and dedifferentiated. Their frequency per plate varied from 5 to 40%. Most of these grains decreased in size and regained their cytoplasm and granular nature in 4–5 days. Many such grains remained abnormally enlarged and empty. Some showed deposits of starch grains later.

(ii) Medium enlarged pollen with dense cytoplasm. Androgenesis was initiated mainly in this type of pollen.

(iii) Small un-enlarged pollen grains which did not respond to culture. Most of them appeared plasmolyzed.

A large percentage of pollen filled with starch grains rarely underwent androgenic initiation, but, occasionally some showed initiation of division and after a few divisions, did not proceed further. Pollen undergoing initiation of embryogenesis acquired a highly granular appearance.

Early microspore divisions in anthers plated at uninucleate and binucleate stages, were initiated between 2nd and 6th day after culture. Out of 400 anthers scored, 121 had dividing pollen grains, their number in an anther varying from 2 to 37. Altogether, 697 androgenic pollen were observed, and of these, 178 were in early division stages, the rest being in advanced multicellular stages. The specific pathways of origin of the latter could not be classified as they had already passed the identifiable stages. Three modes of development were observed.

Pathway I

Initiation occurred in the uninucleate pollen grain by equal division of the single nucleus. After the first pollen mitosis, the two equal-sized daughter nuclei moved apart and underwent consecutive mitotic divisions to give rise to a number of free nuclei (plate 1, A, B and C). This pathway was the most frequent (71.9%, table 1).

Pathway II

Initiation occurred in the binucleate pollen grain where the differentiation of the vegetative and generative nuclei had occurred already *in vivo*. The large diffusely staining vegetative nucleus divided to give rise to the multinucleate pollen while the compact and smaller generative nucleus remained inactive. This type of development was found in 43 (24.1%) pollen grains (plate 1, D and E). The inactive generative nucleus was observed to be located diametrically opposite to the germpore.

Pathway III

Initiation occurred in the binucleate pollen grain where the smaller generative nucleus divided first, followed by the division of the vegetative nucleus and the derivatives of both contributed to the development of the callus. This pathway was the least frequent (3.9%) (plate 1, F, G and H). In this type, the dividing generative nucleus was situated close to the germpore (plate 1, G) or sometimes lateral to it (plate 1, H).

Development and Release of Pollen Callus

After the first few divisions, further divisions within the microspore were independent and asynchronous. Upto 16 or rarely more free nuclei were observed in the

Table 1 *Number of androgenic pollen observed and the frequencies of pollen in the three pathways of androgenic initiation*

Total no. of androgenic pollen	No. of pollen showing division			No. of pollen in advanced stages
	P I	P II	P III	
697	128	43	7	519

multinucleate pollen grain before wall formation occurred. In the multinucleate grains undergoing the third route of androgenic development, smaller nuclei appearing to be derivatives of the more densely staining generative nucleus could be clearly observed. Simultaneous segmentation and wall formation occurred in grains cultured for 12 to 20 days. Further divisions resulted in the formation of enlarged multicellular pollen grains compactly filled with more than 50 cells (plate 1, I, J and K).

The exine of the multicellular pollen ruptured at one or more sites around the 20th day after plating (plate 1, L, M, N, O and P). Pollen callus which emerged gradually from the disintegrating exine, was entirely released from the exine in 6 to 8 days after the emergence of the first cell. In the pollen grains which were originally released into the medium at the time of anthesis, this whole process from initiation of nuclear divisions and release of callus took between 25 to 30 days. However, in the pollen grains held within the anther cavity, development was faster and in 25 to 30 days, relatively much larger calli were formed.

In several instances, the multicellular mass of cells was not released out of the exine. These remained without undergoing further development and presumably degenerated later as has been reported previously (Qu & Chen 1984). Calli produced from pollen held within the anther were of more organized compact appearance while the calli formed from the pollen released into the medium looked more disorganized during their early growth phase.

Fragmentation of Calli

As early as 26th day after inoculation, the calli released from inside the anthers were found to undergo lobing (plate 1, Q). Some of them broke off and these fragments then developed as independent calli. In some cases, these appeared to develop a nodular organized nature and were pro-embryoid-like in structure (plate 1, R). Released calli kept their individuality and no coalescing among these was observed. No recognizable suspensor was observed in these early stages of callus formation connecting it to the anther wall.

Polyploidization of Anther Callus

During early microspore divisions, the metaphases observed in the pollen were all haploid and when multicellular pollen were squashed the callus cells showed haploid complements. However, when calli released from the exine were examined, diploid and polyploid cells were observed. In a young callus removed 32 days after inoculation, haploid, diploid and polyploid cells were observed side by side and in some cases single polyploid cells were observed surrounded by diploid and haploid cells.

Discussion

The number of anthers responding to androgenic development among the 400 anthers scored was about 30% in this study which is close to that earlier reported (25%) in another *japonica* variety, Tainan 5 (Chen & Chen 1980) and very high compared to the general response of rice varieties to anther culture (Raina & Iyer 1974). However, considering the fact that about 400 to 500 pollen grains were present in each anther in this variety, and that the maximum number of multicellular pollen observed in an anther was 37, these observations show that very few of the pollen are initiated into the androgenic pathways. More intensive studies to identify the factors that can trigger a larger number of microspores to undergo androgenic development are essential to enhance the usefulness of this technique. Many of the pollen grains which become enlarged indicating an initial response to culture remained as such and undivided. So it also appeared that a number of grains which underwent a few initial divisions did not attain further development later on. What culture conditions resulted in such inhibitory effect could not be understood. It may be that among the competent pollen showing early divisions, only some are fit enough to continue sustained division and development.

Within 48 hr of inoculation the pollen grains exhibited a trimorphism of response to culture both morphologically and physiologically (plate 1, S & T). The highly enlarged, vacuolated and dedifferentiated cells decreased in size over a period of 2 to 3 days and regained their cytoplasm. Whether division occurred in these grains later could not be ascertained as after this phenomenon of dedifferentiation and 'redifferentiation', they were similar in all appearance to the original medium enlarged category of grains.

Of the three pathways of androgenic initiation, the first where the single nucleus before its differentiation into the vegetative and generative nuclei, gives rise to the multicellular pollen through equal divisions, is the most frequent. This cytological evidence could be correlated to callus induction data in a previous experiment (Mercy et al. 1984) where callus induction efficiency of uninucleate pollen was about three times that of binucleate pollen in this variety. The second and third pathways occur in binucleate pollen where

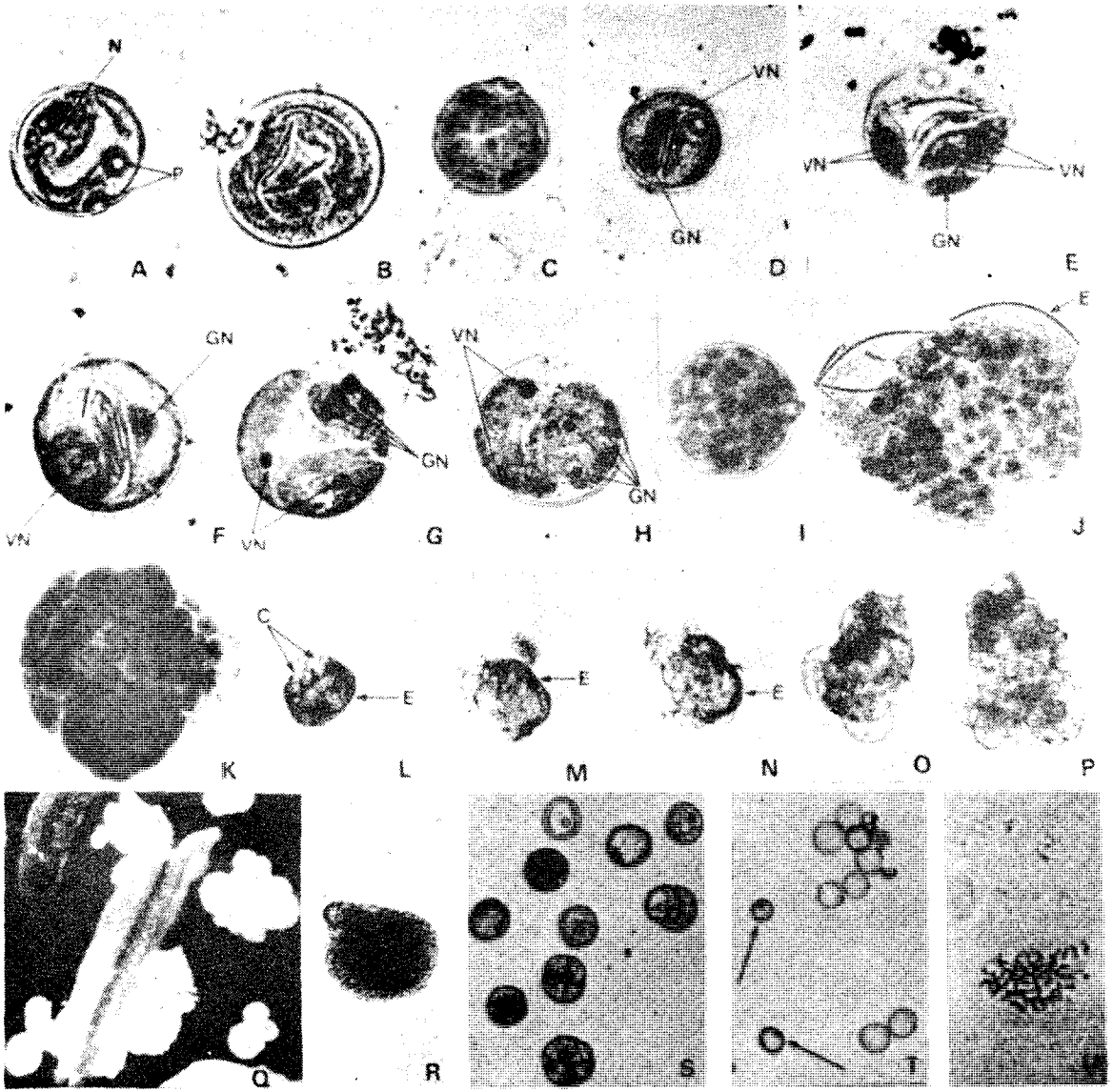

Plate I A–S. A–B, Pathway I; **A,** uninucleate pollen; **B,** pollen with four equal nuclei; **C,** multinucleate pollen; **D–E,** Pathway II; **D,** binucleate pollen with large vegetative nucleus and smaller generative nucleus; **E,** 5 nucleate pollen with four VN and one GN; **F–H,** Pathway III; **F,** large VN and dividing GN; **G,** two VN and three GN; **H,** two VN and several derivatives of GN; **I,** multicellular pollen 16 days after plating; **J,** multicellular grain with exine broken open under pressure and callus cells exposed; **K,** callus 32 days after plating; **L–P,** release of callus sequential shots of same pollen grain 19, 20, 22, 25 and 40 days after plating respectively. **L,** rupture of exine and emergence of callus cells; **M, N** and **O,** gradual disintegration of exine; **P,** released callus; **Q,** lobing of calli prior to fragmentation; **R,** organized pro-embryoid-like structure; **S,** A group of androgenic pollen showing division; **T,** Grains showing differential response to culture; **U,** One dividing polyploid cell.
C callus cells; E exine; N nucleus; P germpore; GN generative nucleus; VN vegetative nucleus.
Figure A–J (×400); **K** (×63); **L–P** (×200); **Q** (×25); **R–T** (×100); **U** (×1000)

differentiation into vegetative and generative nuclei had already occurred. The contributions of vegetative and generative nuclei to pollen callus formation have been studied previously in rice by Chen and Wu (1983) and Qu and Chen (1984).

In these studies, the generative nucleus formed the multicellular pollen through divisions either alone or in conjunction with the vegetative nucleus. The initial divisions of the generative nucleus were always anticlinal according to Chen and Wu (1983), and formation of secondary vegetative and secondary generative nuclei also was observed by them which either contributed to the formation of multicellular pollen units or degenerated. However, we could not confirm these results nor did we observe the formation of secondary vegetative or generative nuclei. In the third pathway, where generative nucleus took part in multicellular pollen formation along with vegetative nucleus, we found that the dividing nucleus generally remained close to the germpore or sometimes lateral to it (plate 1, G & H) whereas *in vivo,* its normal position is diametrically opposite to the germpore (plate 1, D). After the first division of the generative nucleus which was anti-clinal, later divisions were not always anticlinal or synchronous. Conclusive evidence for the formation of the multicellular pollen exclusively from generative nucleus was not obtained from our study.

When generative nucleus did not contribute to callus formation, it could be seen close to the exine opposite the germpore, during the first few free nuclear divisions of the vegetative nucleus. No degeneration of the generative nucleus was observed at these stages. In pollen grains undergoing development in the third pathway, the derivatives of vegetative and generative nuclei could be identified conclusively by their size and staining during the first several divisions during which both types of nuclei divided independently and non-synchronously. Pollen where all the nuclei appeared similar in size and staining ability, perhaps arose from uninucleate grains by a symmetrical initial division. When the development advanced further it was not possible to identify the origin of multicellular pollen or callus i.e., whether it was derived from uni- or binucleate pollen and no distinction could be made among the three pathways.

Though the release of callus from the exine was closely followed from the living grains in culture plates, no contraction of multicellular pollen prior to the breakage of exine was observed as reported earlier by Qu and Chen (1984). The release of callus was a gradual process taking 6 to 8 days (plate 1, L–O) from the emergence of the first cell and the remnants of the disintegrating exine could be observed attached to the new callus for some time.

An interesting observation was the very early fragmentation exhibited by many of the pollen calli. This showed that all the calli which are recovered 35 to 40 days after plating may not necessarily be originating from individual pollen grains and chances are that several calli may be produced from one developing grain as the fragments get easily dispersed in the liquid medium (plate 1, Q). This may not be important when pure lines are used for culture but the implications of this observation are more pertinent when the parent material is heterozygous, as this would give rise to distorted progeny ratios in the genetic analysis of anther-culture-derived plants utilized for linkage and gene mapping studies. Such distorted progeny ratios were also observed by Orton and Bowers in Brassica (1985) when they found that populations derived from microspores do not necessarily represent a random gametic array even when linkage was absent and significant over-representation of one fast migrating class of gametes was observed in their results. However, one way to minimize the fragmentation of calli might be the transfer of the calli for regeneration as early as possible. The liquid medium allows the fast dispersion of the calli and their fragments thereby allowing more and more competent pollen grains to develop without being crowded within the anther. This also prevents the calli originating from different pollen grains from coalescing together. However, though one can be sure that each callus is originating from an individual pollen grain, it is also possible that several might come from the same through fragmentation.

Suspensor-like connection between the microspore and the anther wall in the early microspore division stages as reported by Guha et al. (1970) was not observed in the present study. However, calli developed from the pollen released into the medium had a more disorganized structure and their growth was also slow as compared to the calli developed from pollen held within the anther walls which was faster and which had better, more compact and organized appearance. This points out to an essentially nutritional role of the anther wall in the formation of pollen callus. However, this may be through diffusion rather than through establishment of actual cellular connection.

A previous study by the authors (Mercy & Zapata 1986) on the ploidy levels of a random sample of 209 anther-culture-derived plants indicated that such plants were mostly haploids (42.0%) and homozygous diploids (56.5%) with a small percentage (1.5) of polyploids (see Raina & Iyer 1974). However young calli removed 32 days after anther inoculation and cytologically analyzed showed the presence of a relatively large number of polyploid cells (plate 1, U) along with haploid and diploid cells. This indicated that doubling of chromosomes and further polyploidization were already occurring in 10–12 days old callus cells. Wide variation in chromosome number in 50–55 days-old pollen calli of F_1 hybrid plants of rice has

been reported earlier by Oono (1981). Based on our cytological observations we could not correlate the polyploidization with the uninucleate or binucleate stage of the pollen. Neither could we get any evidence for nuclear fusion, though it can easily occur during the early free nuclear stage as postulated by Chen and Wu (1983) rather than at later stages which would involve dissolution of the cell walls. Polyploid complements were not observed in early divisions of the multicellular pollen grains and often isolated single polyploid nuclei appeared among haploid and diploid cells in the callus. From our studies, it appears that polyploidization is possible due to endomitosis or endoreduplication. In the competition for growth during later development, the faster-growing haploid and diploid cells in the callus might overcome the possibly slower growing polyploid cells and this might explain to some extent the very low percentage of polyploids obtained among regenerated plants.

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