PHP, which rarely has these mechanisms. Pollen morpholohy is related to water content. PDG have furrows, whilst PHG are devoid of these harmomegathic device.

FRANCHI, G. G., NEPI, M., DAFNI, A. & PACINI, E. 2002. Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. **Plant Systematics and Evolution** 234: 211-227.
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The extracellular lipids and lipid-binding proteins of pollen grains - their biosynthesis and roles in pollination

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The extracellular matrix of the pollen grains of many entomophilous plants is covered with a sticky substance known as the tryphine, or pollen coat. We have characterised the pollen coat components in plants of the Brassicaceae, including rapeseed (*Brassica napus*) *and Arabidopsis thaliana*. The pollen coat in these species is mostly made up of a unique mixture of lipids that includes acylated compounds such as sterol esters and phospholipids. These lipids are characterised by their unusually high degree of saturation – they typically contain 70-90% saturated acyl residues such as myristate, palmitate and stearate. The major sterol components of the pollen coat are acyl esters of stigmasterol, campesterol and campestdienol respectively. The second major pollen coat component is a group of proteins that we term pollenins. The pollenins are derived from larger proteins, called oleo-pollenins, that are synthesised in the anther tapetum. Oleo-pollenins contain an N-terminal domain that is similar to the central hydrophobic domain of seed oleosins. This oleosin-like domain is removed by a specific peptidase after the oleos-pollenins are released into the anther locule following tapetal apoptosis. The mature pollenins are made up of a diverse series of repetitious motifs that are characteristic of structural proteins, rather than enzymes. In this talk, I will describe the mechanism and localisation of the biosynthesis of the pollen coat protein and lipid components and their relocation to the pollen wall. I will then discuss the function of the oleosin-like domain as a novel targeting signal. Finally, I will also discuss the possible roles of pollenins and the pollen coat lipids in adhesion to insect vectors and in pollen-stigma interactions both during and following fertilisation.

Pollen ontogeny in the Nymphaeales

Osborn, J. M.; Schwartz, J. A.; Gutman, B. L.; Melroe, N.A.; Ingraham, A. M.; Taylor, M. L.; Strandquist, J.N.; Hudson, P. J.; Miesner, J.M.; Schoenekase, A. N.; Thiemann, T. & Doores, A. S.

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The Nymphaeales, or water lilies, have a cosmopolitan distribution in fresh water habitats and comprise two families and eight genera: Nymphaeaceae (*Victoria, Euryale, Nymphaea, Ondinea, Barclaya, Nuphar*) and Cabombaceae (*Cabomba, Brasenia*). Water lilies are widely regarded to be among the most primitive flowering plants, as recent phylogenetic studies have consistently indicated that either *Amborella*, or *Amborella* plus Nymphaeales is the sister group to the remaining angiosperms. Although studies of pollen development provide important data for assessing phylogeny, little is know about these characters in the Nymphaeales.

In this presentation, data on pollen and another ontogeny will be described and reviewed for all genera of Nymphaeales, with emphasis on the taxa that have been studied with combined scanning electron, transmission electron, and light microscopy. Anthers at the sporogenous tissue, microspore mother cell, tetrad, free microspore, and mature pollen grain stages will be documented. Events including the deposition of a microspore mother cell coat, a callose 'special' wall, a primexine, and the sporoderm layers will be discussed.The tetrad stage proceeds rapidly, and the tetrads of most genera are of the tetragonal type. In addition to this tetrad configuration, several genera also co-produce tetrahedral tetrads. The mature pollen grains of most genera occur as monads; however, in

Victoria grains are held together in permanent tetrahedral tetrads. In *Euryale*, the production of pollen monads is typical, but the formation of permanent tetrads and dyads has also been observed. Significant exine deposition, including formation of the columellae and tectum occur during the tetrad stage. Pollen of all genera has a columellar infrastructure, with variation occurring in columellae size and ultrastructure. Development of a series of prominent tectal/columellar microchannels occurs in *Cabomba*. Exinous microchannels are also produced in the pollen of *Nuphar* and *Victoria*. The endexine lamellae and a foot layer form during the free microspore and early pollen grain stages. Pollen grains of only two genera possess major sculptural elements: supratectal rods in *Cabomba* and spines in *Nuphar*. The spines of *Nuphar* are ultrastructurally different and have an earlier ontogenetic origin than the scultural rods of *Cabomba*, or the minor tectal ornamentation of other water lilies. The principal characters of anther ontogeny discussed will include the number and size of anther wall layers, changes in tapetum morphology, and timing of tapetum dissociation. An amoeboid tapetum has been observed for the first time in several nymphaelean genera.

Water lilies are also characterized by diverse pollination mechanisms, including beetle, bee, fly, and wind syndromes, as well as cleistogamy. Because pollination syndromes have been well documented within the Nymphaeales, the group provides as excellent system to investigate functional correlations between pollen developmental characters and pollination ecology. In addition, given the basal position of Nymphaeales, the group provides the opportunity to investigate the early evolution of pollination ecology and associated reproductive characters within angiosperms. For example, *Cabomba caroliniana* is fly-pollinated and mature pollen grains are coated with copious amounts of tapetally derived pollenkitt, that is stored within the exinous microchannels. The ontogenetic timing and ultrastructure of these microchannels appear to be adaptation for pollination. The adaptative significance of such developmental characters, along with their importance in examining systematic and phylogenetic relationships of the Nymphaeales will be discussed.

Session a2

SIGNALLING IN POLLEN DEVELOPMENT, STRESS-INDUCED MICROSPORE EMBRYOGENESIS AND POLLEN GERMINATION

Signal transduction by MAP kinases in pollen germination

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Mitogen-activated protein (MAP) kinases are highly conserved in eucaryotes and have been implicated in the transduction of a variety of extracellular signals involved in development and stress response. Downstream targets include transcription factors but also other cellular structures such as the cytoskeleton. MAPKs are part of a three-partite signalling module consisting of a MAPKKK (or MEK2), a MAPKK (or MEK) and a MAPK. Eucaryotic genomes contain gene families for all three members of the module, including plants.

We have isolated a number of MAP kinase genes from tobacco and alfalfa. The tobacco MAP kinase NTF4 accumulates in mature, dry pollen as an inactive kinase. Upon rehydration the NTF4 kinase is rapidly and transiently activated, and is again inactivated well before pollen tube emergence (Wilson et al. 1997). Water alone is sufficient to activate this kinase. NTF4 or a very similar MAPK is also involved in hypoosmotic stress signaling in somatic cells (Cazale et al. 1999).

We have now found that the highly related MAP kinase SIMK is also expressed and activated in pollen by hydration and that both MAPKs are regulated by the MAPKK MEK2. Transient transformation of pollen with a kinase-negative mutant version of MEK2 indeed inhibited pollen germination (Voronin et al. 2004).

WILSON, C., VORONIN, V., TOURAEV, A., VICENTE, O. & HEBERLE-BORS, E. 1997. A developmentally regulated MAP kinase activated by hydration in tobacco pollen. **The Plant Cell** 9: 2093-2100.
CAZALE, A., DROLLARD, M., WILSON, C., HEBERLE-BORS, E., BARBIER-BRYGOO, H. & LAURIERE, C. 1999. MAP kinase activation by hypoosmotic stress on tobacco cell suspensions: towards the oxidative burst response. **Plant J**. 19: 297-308.
VORONIN, V., AIONESI, T., LIMMONGKON, A., BARINOVA, I. L., TOURAEV, A., LAURIÈRE, C., CORONADO, M.-J., TESTILLANO, P.S., RISUENO, M.-C, HEBERLE-BORS, E., WILSON, E. 2004. The MAP kinase kinase NtMEK2 is involved in tobacco pollen germination. **FEBS Letters** 560: 86-90.

MAPKS are induced and entry into the nucleus during stress-induced pollen embryogenesis and pollen development

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Plant developmental processes involve both proliferation and differentiation events, cellular and nuclear dynamics varying during developmental processes and in response to factors like stress conditions. Mitogen-activated protein kinases (MAPKs) are involved in the signalling of extracellular stimuli in eukaryotes, including plants. The reversible phosphorilation of MAPKs plays a pivotal role of activating specific cytoplasmic and nuclear targets which are the final effectors of the cellular responses. Different MAPKs have been shown to be expressed during plant cell proliferation and developmental processes such as pollen development and pollen embryogenesis.

Extracellular signal-regulated kinases (ERKs) proteins belonging to the MAPK family are involved in both cell proliferation and differentiation in mammals, through phosphorilation pathways. ERKs homologues have been recently identified in tobacco cell suspensions, being activated in response to plant defence. In this work the expression and subcellular localization of ERK1/2 homologues, and MAPK-active forms were studied in two pollen developmental programmes: stress-induced pollen embryogenesis (which involved an initial proliferation), and pollen maturation (which involved a differentiation) by immunoblotting,, immunofluorescence and immunogold labelling, in various plant species. Quantitative studies of the labelling obtained were also performed.

The results were similar for all the species studied showed that during pollen development and embryogenesis. ERK and phospho-MAPK labelling signals increased, specially in the nucleus, indicating that the progression of differentiation and proliferation was accompanied by an increase in the expression of ERKs and MAPK activation together with a translocation to the nucleus. The combination of ultrastructural cytochemistry and immunogold for RNA and phosphorylated proteins indicated that the nuclear sites housing these MAPKs were areas of the interchromatin region enriched in RNA and phosphoproteins, including clusters of interchromatin granules. This could suggest a role of these MAPKs in the early events of activation of the transcription and processing machinery, via phosphorylation, which subsequently would be recruited to the transcription sites. The association of the nuclear localization of MAPKs with the stress-induced progression through the cell cycle and the commitment towards differentiation in the two pollen developmental processes can be correlated.

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CORONADO, M. J., GONZÁLEZ-MELENDI, P., SEGUÍ, J. M., RAMÍREZ, C., BARANY, I., TESTILLANO, P. S. & RISUEÑO, M. C. 2002. MAPKs entry into the nucleus at specific interchromatin domains in plant differentiation and proliferation processes. **J. Struct. Biol**. 140: 200-213.

Promoter expression of a putative pollen monosaccharide transporter in *Petunia hybrida* and characterisation of a transposon insertion mutant

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The interaction between style and a developing pollen grain is a remarkably process which is only poorly understood and mainly studied from a non metabolic point of view. The astonishing speed of pollen tube growth and the extreme length of this single cell apendix requires large amounts of metabolites for energy consumption and de novo cell wall biosynthesis. A vital compound for the development and growth is sucrose. In pollen grains of *Petunia hybrida* towards maturity, carbohydrates are accumulating in the form of starch. These polysaccharides will be used as an energy source for pollen tube growth. After germination of the pollen grain at the stigma, the pollen tube has to transverse distances in the pistil often thousands times the diameter of the pollen grains to deliver the male gametes to the embryo sac for fertilisation (Cheung, 1996). Therefore, a great amount of energy will be needed for the formation of the long pollen tube, which requires a high level of sugar import (Schlüpmann et al., 1994). The sugar final destination, however, requires translocation from the anther, stigma and stylar apoplast over the pollen tube membrane. It is known that usually the apoplastic unloading of sugars into the sink tissues involves an hydrolysis of sucrose into glucose and fructose by means of an acidic invertase, subsequently followed by an active uptake of these monosaccharides via monosaccharide-proton-symporters (Roitsch & Tanner, 1996). In terms of sink-source relations, pollen and growing pollen tubes should be regarded as a strong sink.

From *Petunia hybrida* a pollen-specific putative monosaccharide transporter designated PMT1 has been identified in an earlier paper. (Ylstra et al., 1998) This work comprises an "in depth" analysis and characterisation of PMT1 in the context of pollen development using the GUS reporter gene and an insertion mutant.

The promoter of the pollen specific putative monosaccharide transporter gene from *Petunia hybrida* (*pmt*1) has been isolated by inverse-PCR and sequenced. Analysis of plants transformed with the promoter-GUS fusion confirmed the specificity of this gene, belonging to the late pollen-specific expressed genes. GUS activity was detected even after 24 h of *in vitro* pollen germination, at the pollen tube tip.

To elucidate the importance of *pmt1* for gametophyte development and fertilisation a mutant plant containing a trasposon insertion in the *pmt*1 gene was isolated using *dTph1* transposon tagging-PCR based assay. The *pmt1* mutant contained a *dTph1* insertion in position 1474 bp of the transcribing part of the gene, before the last two transmembrane spanning domains. Analysis of the progenie of the heterozigous mutant after selfing revealed no alterations in pollen viability and fertility. Mature pollen grains of a plant homozygous for the transposon insertion were able to germinate *in vitro* in a medium containing either sucrose, glucose and fructose, which indicates that *pmt*1 is not essential for pollen survival. Several explanations for these results will be discussed in this presentation.

CHEUNG, A. Y. 1996. The pollen tube growth pathway: its molecular and biochemical contributions and responses to pollination. **Sex Plant Reprod.** 9: 330-336.
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WILLIAMS, L. E., LEMOINE, R. & SAUER, N. 2000. Sugar transporters in higher plants-a diversity of roles and complex regulation. **Trends Plant Sci.** 5(7): 283-290.
YLSTRA, B., GARRIDO, D., BUSSCHER, J. & VAN TUNEN, A. J. 1998. Hexose transport in growing pollen tubes and characterization of a pollen specific putative monosaccharide transporter. **Plant Physiol**. 118: 297-304.

Characterization of proteins secreted during maize microspore culture: arabinogalactan-proteins (AGPs) stimulate embryo development

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To study molecules secreted from cultured plant cells that promote development maize microspores were transfered into culture and the conditioned media were collected over time and analysed. Electrophoresis indicated that both non-glycosylated and glycosylated proteins including arabinogalactan-proteins (AGPs) appeared into the medium and their concentration increased during the time of culture. The development of embryos was correlated with the presence of specific extracellular proteins, using an experimental system based on a tunicamycin inhibition test. In addition, a precise protein analysis was conducted using MALDI-TOF and ESI-MS-MS techniques. These approaches have allowed the identification of 5 other types of proteins: a cell wall invertase, two thaumatin isoforms, one 1-3 beta-glucanase and two chitinase isoforms. Altogether these experiments and results open ways for research aimed at understanding which molecules stimulate embryo formation. Moreover, AGPs may be used to stimulate the development of microspores (pollen embryogenesis) prepared from non-responsive genotypes. The possible role of the secreted molecules will be discussed and a parallel will be made between the development of an embryo in liquid medium and *in planta*.

Signal transduction in pollen: Ca^{2+}, phosphoinositides and calmodulin crosstalk in the pollen tube apex

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Pollen tube growth relies on an extremely fast delivery of new membrane and wall material to the apical region where growth takes place. Despite the obvious meaning of this fact, the mechanisms that control this process remain very much unknown. A tip-focused [Ca2+]c gradient is known to play a central role in the regulation of pollen tube growth and modulation of the [Ca2+]c concentration results in changes in the rate and direction of growth. One of the targets of this Ca2+ signalling pathway was therefore claimed to be the secretory pathway and similar claims were made for phosphoinositides. We investigated the role of phosphoinositides and [Ca2+]c in membrane traffic in pollen tubes and to which extent, if any, the two signalling pathways are connected. It was found that both modulation of phosphoinositides and increase in [Ca2+]c stimulate secretion but interestingly, PIP2 and IP3 had different effects, thus suggesting the existence of different target molecules.

Endo-exocytosis seems to be modulated also by a crosstalk between calmodulin (CaM) and cAMP. CaM activity exhibits a tip-focused gradient, similar to the distribution of cytosolic free calcium $[Ca^{2+}]_c$ which oscillate with a period similar to $[Ca^{2+}]_c$ (40-80 sec). Changes in CaM activity within the dome of the pollen tube apex induced reorientation of the growth axis suggesting that CaM is involved in the guidance mechanism. CaM activity was found to be modulated by changes in cAMP levels, indicating that the action of this protein is not solely dependent on $[Ca^{2+}]_c$ but on a crosstalk with other signalling pathways.

3D confocal and electron microscopy imaging define the dynamics and mechanisms of diploidisation at early stages of barley microspore-derived embryogenesis

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At specific immature stages, the pollen developmental programme can be switched towards cell proliferation, haploid embryogenesis and plant regeneration under appropriate culture conditions –a process known as androgenesis or microspore-derived embryogenesis. As a consequence to a spontaneous diploidisation throughout this process, homozygous doubled-haploid plants are formed. In modern plant breeding, this technology is employed to generate populations which consist of true-breeding individuals and have the potential to encompass the entire genetic variability of an initial cross combination within a minimal scope of plants. Furthermore, this technology can be coupled with *Agrobacterium*-mediated transformation to produce T_0-plants homozygous for the gene transferred.

We have developed a technical advance to determine the ploidy level of individual nuclei within androgenetically developing pollen by using specific fluorescent probes. This study was performed in barley throughout early androgenic development in order to determine the timing of diploidisation. We found that confocal 3D-imaging of these preparations was a reliable method. By using the number of nucleoli and morphometric data such as the measure of the nuclear area (to estimate the nuclear size) as markers of the ploidy level we observed that diploidisation starts at very early stages in the cultures. Reconstruction of 3D-images of entire pro-embryos and the observation of cross and longitudinal sections across stacks of optical sections together with correlative light and electron microscopy provided unambiguous snapshots of nuclear fusion as the mechanism of genome doubling in the process of microspore-derived embryogenesis.

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Exclusive formation of true-breeding transgenic T_1-plants after *Agrobacterium*-mediated transformation of barley pollen cultures

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Methods used to identify and clone genes and promoters which are of scientific interest or have putative relevant function for plant breeding are becoming increasingly powerful. Therefore, reliable and efficient systems for genetic transformation of important crops are necessary for comprehensive functional gene analyses as well as for crop improvement approaches. Based upon the huge regeneration potential of pollen cultures, a novel method has been developed for *Agrobacterium*-mediated transformation of barley. Although a few T_0-plants which are homozygous (true-breeding) for the gene transferred can be directly generated by this method, these individuals cannot be phenotypically distinguished from their hemizygous sister plants. As a consequence, tedious and time-consuming segregation analyses are necessary to identify these rare homozygous transformants. Furthermore, many of the primary transgenic plants generated via transformation of barley pollen cultures are haploids. These haploid regenerants appear to be useless in the first instance, because they do not set seed.

Following a novel concept, the haploid primary transgenic regenerants were routinely identified by flow cytometry at an early stage of development and subsequently subjected to induced genome doubling. As a result, more than 90 percent of these plants showed partial or full seed set. Although the inflorescences from many colchicine-treated plants appear to be ploidy-chimaeric, all caryopses obtained must be homozygous for the transgene, since fertile flowers can only be formed from diploid (doubled haploid) cellular origines. Thanks to stable transgene expression over generations, exclusive formation of T_1-plants which are true-breeding with regard to the gene introduced will substantially contribute to increased efficiency, reproducibility and reliability of overexpression, knock-out and mutant complementation experiments

Monitoring of *in vitro* cultured microspores switched to embryogenesis in *Olea europaea* L.

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Genetic improvement through conventional methods of breeding in woody plants involves many generations, and may take several years before any meaningful results are obtained and has still not given fruitful results. This has led to more emphasis on the use of *in vitro* techniques especially anther and microspore culture for genetic improvement of woody species like olive (*Olea europaea* L.). Olive tree is a woody and cultivated species, main product in Spain. The olive oil has a social importance and a good future due to consumer's necessities.

Searching for convenient morphological criteria to correlate anatomy of the flower bud with microspore developmental stage: For high frequency of haploid production, the appropriate stage of microspores is critical and it varies with species, and thus it is necessary to identify the appropriate stage of microspores for haploid plant production. (Bueno *et al.* 2002). To study the stage of the microspore in olive, directly from the trees growing in the field , its has taken branches with floral bud from the World-wide Collection Cultivars from Olive tree of Cordoba (Spain) in different zone of the tree. Numerous preparations with Aceto-Carmine and DAPI have been made, to be able to find parallelism between the morphology of floral buds and the stage of the microspore. Great asincrony has been observed, like in most of the woody species. Inside anthers extracted from flower buds with a similar size and color, microspores at uninucleate stage were obtained, whereas other floral bud with these same characteristics contained different stages, from tetrad until polen. During the gametophytic development, the initial stages of the material isolated from the anthers show very young microspores released from tetrads- after meiosis of the pollen mother cells- which are characterized by small size, weak autofloresecence of the wall under UV and a large and centrally located nucleus. In late-stage microspores, the nucleus is slightly condensed and located mostly near the polen wall. Microspore mitosis is asymmetric and produces nuclei that can be easily distinguished after staining with DAPI. Then, the generative nucleus is small, condensed and appears intensely stained; whereas, the vegetative nucleus is large, diffuse and appears weakly stained.

Monitoring in vitro formation of microspore-derived multinuclear structures: The sporophytic pathway *in vitro* will be determinated by the genotipe. When late vacuolate microspores or young bicellular pollen grains are cultured under stress conditions, they are switched from their naturally programmed pathway for gametophytic to sporophytic development. Then, these microspores start dividing in a different way than they do *in vivo*. There is a symmetric divisions with several nuclei 2,4,8...leading to embryoid or proembryo formation. The initiation of embryogenesis it is possible to observed in the multicellular structures that are in progress. The obtained pure lines, will be used for future genetic analysis and breeding programs. These results will have advantageous implications at scientific, social and economical levels.

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Use of gametic embryogenesis for *Citrus* improvement

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Citrus with 104,505,157 Mt in 2002 (FAOSTAT database) is the main world fruit crop. Breeding programs in *Citrus* aim to obtain new varieties with a shorter juvenile period, increased yield, a longer ripening season, regular bearing, improved fruit appearance and quality and, above all, without seeds.

Haploidy advantages and pollen biotechnology can represent a powerful tool in *Citrus* breeding (Germanà, 1997), with potential uses in mutation research, selection, genetic analysis and genetic transformation. Breeders and plant geneticists are also interested in obtaining homozygousity in woody plants, generally characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, self-incompatibility. This interest justifies the need to conduct further research thereby increasing our knowledge of gametic embryogenesis with the goal of establishing efficient anther culture protocols in this important agricultural crop (Germanà, 2003).

Anther culture is one of the most interesting systems of plant regeneration through *in vitro* culture, and gametic embryogenesis is one of the most stricking examples of cellular totipotency (Reynolds, 1997).

Androgenesis has been successfully induced in genus *Citrus*, but only in few genotypes. Actually, the improvement of the induction rate and the increase of number of genotypes responding to androgenesis are important with respect to enhancing haploidy application in *Citrus* biotechnology and breeding. Although androgenesis research made great progress in recent years, several aspects of this phenomenon still remain unclear, particularly the induction process of androgenesis and the factors that control it. The identification of the inhibitory and stimulatory factors are fundamental especially for recalcitrant species like *Citrus*.

From anther culture of *Citrus* and its relatives, haploid, but also diploid and above all triploid calli, plantlets and plants have been obtained. Ploidy analyses of androgenic regenerants, enabled us to demonstrate that the largest percentage of them are triploids and not haploids or doubled-haploids as expected, opening the way to a fast new, innovative and promising tool to obtain seedlees triploids in *Citrus*. The importance of triploids in *Citrus* improvement stems from the seedlessness of their fruits; and the recovery of seedless varieties is one of the main goals in *Citrus* varieties breeding, because they are strongly required by the fresh fruit market.

Genetic analysis using isozyme analyses and microsatellite markers showed that the regenerants are homozygous and produced by gametic embryogenesis.

This report summarizes the current status of research on androgenesis in *Citrus*.

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Session a3

POLLEN FOR IN-VITRO PRODUCTION OF HAPLOIDS

The relationship between induction of embryogenesis and chromosome doubling in microspore cultures

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Chromosome doubling of haploid plants has been a major concern relative to their use in plant breeding, genetics, mutation and transformation. The ideal time to double the chromosome number would be the haploid uninucleate cell or microspore stage following meiosis. The microspore stage is most often used to induct embryogenesis for haploid plant production as it can provide a large number of partially synchronized cells. The uninucleate stage can be induced by stress or anti-microtubule agents to provide embryo induction. The slightly