FORMATION OF UNREDUCED POLLEN IN *ARABIDOPSIS THALIANA*

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INTRODUCTION

Sexual polyploidization, as a result of 2n gamete formation, is thought to be the main process involved in the origin of polyploid plants (Bretagnolle & Thompson, 1995). These gametes, possessing the somatic rather than the gametophytic chromosome number, can be formed either during microsporogenesis (2n pollen, big pollen, diplandroidy) or megasporogenesis (2n eggs, diplogynoecy). In general, unreduced gametes are diploid; however, triploid and tetraploid gametes have been described on diploid sporophytes and referred to as 3n and 4n gametes, respectively (Veilleux, 1985).

Unreduced pollen can be formed by pre- or post-meiotic chromosome doubling (endo-reduplication), but are mainly the result of a meiotic disfunctionment, which leads to a mitosis-like non-reduced division. Although 2n pollen may result from a variety of different meiotic anomalies, the type of 2n gametes produced results from one of two basic restitution processes, namely First Division Restitution (FDR) or Second Division Restitution (SDR). In FDR, the pairing and/or the separation of the homologous chromosomes at anaphase I does not occur, as similar to a mitotic division. However, the second meiotic division occurs normally, with the two sister chromatids of each chromosome moving to the opposite poles. In SDR, first meiotic division occurs normally, but the sister chromatids do not separate during the second meiotic division.

The genetic consequences of both FDR and SDR gametes are very divergent and this may be important in the view for potential breeding applications. Both meiotic anomalies result in the formation of two unreduced, instead of four reduced, microspores. However, a 2n gamete produced by FDR will possess two non-sister chromatids, maintaining the level of heterozygosity and epistasis of its parents, whereas a 2n gamete produced by SDR will posses the two sister chromatids, who are identical from the centromere to the chiasma (if there was recombination), significantly decreasing the parental heterozygosity (Bretagnolle & Thompson, 1995).

As stated by several authors, plant species producing a significant percentage of 2n gametes can have interesting applications in present-day agriculture and crop breeding. For instance, 2n gametes of the FDR type are very efficient in transferring parental heterozygosity and retaining epistatic interactions. In several crops, the use of diploid meiotic mutants that produce 2n FDR gametes is considered as one of the main methods for exploiting hetero-

sis and introgressing wild germplasm traits in tetraploid crops by means of 4*x*-2*x* crosses and reciprocals (Ramanna & Jacobsen, 2003). In alfalfa, this technique has been succesfully employed creating hybrids with higher dry mass contents . Moreover, in lily and tulip it is shown that the induction of 2n gametes can be used for overcoming F1-sterility in interspecific hybrids (Barba-Gonzalez et al., 2006).

Although the genetic determination of 2n pollen production has been studied in great detail for many species, a solid basic control mechanism for all plants has not been found yet. In *Solanum tuberosum*, the meiotic anomaly causing the formation of 2n pollen -a defect in spindle formation- seemed to be the result of one mutant allele, e.g. the *desynaptic* mutant. However, other authors suggest that the formation of a parallel spindle is caused by a dominant allele with a variable expressivity or even by several interacting genes. Indeed, in other species, such as *Trifolium pratense* and *Petunia*, polygenetic control mechanisms, rather than a single mutant allele, are found to be the cause of the meiotic anomalies leading to 2n pollen production. Hereby, the cytological anomalies are under the control of a monogenic recessive allele, whose expressivity depends upon some minor genes and the external environment (Bretagnolle & Thompson, 1995).

In the search for the genetic basis of 2n pollen production, this project focuses on the in depth study of pollen development and meiosis progression in the genetic model species *Arabidopsis thaliana*. By identifying molecular and fysiological factors, contributing to a significant production of 2n pollen in *Arabidopsis* flowers, the genetic control of various types of meiotic non-reduction can be clarified and elucidated.

MATERIAL & METHODS

Pollen ploidy-size correlation

In order to detect plants that produce a significant number of 2n pollen, a variety of methods differing both in technical sophistication and exact objectives, have been developed. The main methods are morphological and flow cytrometric analysis of pollen (Dewitte et al., 2006), progeny analysis and the cytological examination of microsporogenesis (Koornneef et al., 2003).

Morphological examination of pollen by means of size measurements is often the most direct screening method. Because of the relatively close correlation between pollen DNA content and cell volume, the occurrence of giant pollen grains is often used as an indication of 2n pollen production. However, for *Arabidopsis* pollen, only one publication shows this correlation (Altmann et al., 1994) and further evidence is absent.

Therefore, in this study, the *Arabidopsis* pollen ploidy-size correlation is demonstrated by measuring and comparing the diameters, cross-sectional surface areas and volumes of pollen derived from plants of different ploidy levels (diploids and tetraploids). Large numbers of hydrated pollen grains were microscopically imaged and morphologically analyzed, using a Matlab

imaging analysis script. For each ploidy level, at least 250 pollen were analyzed and documented. In addition, for volumetric assays, pollen samples were analyzed by impedance measurements in an electrical sensing zone device (Beckman Coulter Counter Z2).

Giant pollen mutants

The first step in the genetic elucidation of the process of 2n pollen formation in *Arabidopsis* is the search for mutants, producing a significant amount of unreduced pollen grains. In our screens, mutants were first isolated based on the pollen ploidy-size correlation. These giant pollen mutants, then, can be used to isolate inheritable modifications responsible for the unreduced gamete formation by means of progeny analysis, transcriptomics and genetics.

In this project EMS (ethyl methane sulphonate) mutagenized *Arabidopsis thaliana* Col-O wt M2 plants (obtained from Lehle Seeds) were grown under standard conditions. To identify diploid EMS mutants, exhibiting a high frequency of giant pollen production, a double screening method was performed. In the first screening round, whereby each sample contained pollen of five different EMS plants, pollen grains were sized based on a volumetric assay using a dielectric volume measurement device. The threshold mean pollen diameter in these analyses was set at 22,27 µm. When this level was exceeded, pollen were considered significantly enlarged (giant pollen or GP). Samples exhibiting a GP production larger than 5% on a volumetric base were withheld. Because each sample contained pollen of five different EMS mutants, a second screening had to be performed to isolate the GP mutants. Hereby, for each mutant, pollen were collected, microscopically imaged and morphologically analyzed, using a Matlab imaging analysis script. Upon the identification of GP producers, plants were isolated, characterized and seeds were harvested for further progeny analyses.

RESULTS

Pollen ploidy-size correlation

The assays revealed that for all pollen size parameters (transsectional surface area, major and minor axis and volume) there was a significant difference between the analyzed ploidy levels. However, as seen in the surface area and the volumetric histograms (Figure 1), there is a small size overlap between pollen harvested from the diploid and the tetraploid lines.

Based on these measurements, for diploid *Arabidopsis* plants, size thresholds were set above which the pollen are considered anomalously enlarged. Pollen grains having a transsectional surface area larger than 550 μm^2 or having a volume larger 46264.6μm^3 (sphere with diameter 22.27 µm), are called giant pollen.

Spontaneous giant pollen production in diploid *Arabidopsis thaliana* Col-O wt plants was analyzed using the transsectional surface area as pollen size parameter. Frequency of GP formation was generally very low and showed a high variability, not only between individual plants but even between the different flowers of one plant. On the whole, giant pollen production in diploid *Arabidopsis thaliana* Col-O wt amounts to 0.3%.

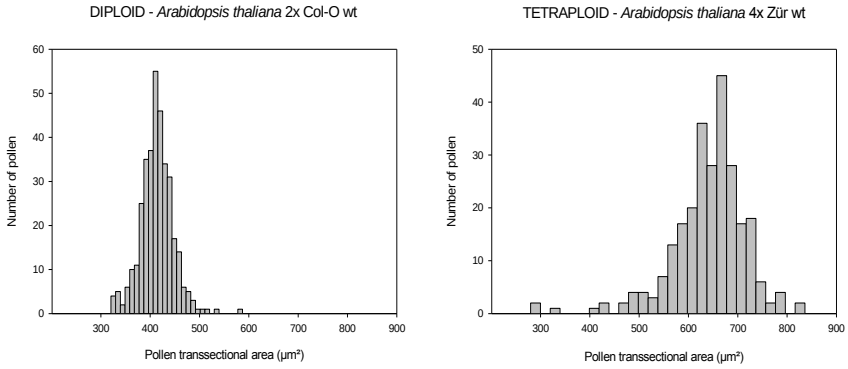
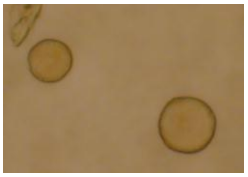


Figure 1: Size distribution of pollen of a diploid and a tetraploid *Arabidopsis thaliana* accession.

Giant pollen mutants



Several interesting mutants were discovered, all producing a significant amount of giant pollen grains. In most mutants, frequency of GP production lies between 1 and 20%. However, in one mutant, EMS 1-1 nr 12, GP production amounts to more than 50%. In another mutant, EMS 1-7 nr 3, the giant particles appeared to be intact or degenerated tetrads of pollen grains, corresponding to the formerly found Quartet mutant.

CONCLUSIONS

In order to detect the formation of unreduced pollen in diploid *Arabidopsis* plants, pollen size can be used as a direct morphological screening criterion. Due to the significant separation between x (from diploid) and 2x (from tetraploid) pollen size distributions, pollen volume and/or pollen transsectional surface area can be used as an easy-to-measure parameter for pollen ploidy determination. In our research, this morphological feature is used to identify EMS mutants, potentially producing significant amounts of unreduced pollen.

The first step in the search for genetic factors involved in 2n pollen production has been achieved. Several mutants producing significant amounts of

giant pollen grains are identified and isolated. Next, to verify if the selected mutants actually produce unreduced pollen, and not solely larger gametes, pollen ploidy analysis has to be performed using flow cytometry. In order to elucidate the mechanism of unreduced pollen formation (FDR or SDR), selected mutants will be submitted to a cytological analysis of microsporogenesis and meiosis. Next, in order to isolate inheritable modifications, responsible for unreduced pollen formation, progeny analysis and backcrosses of these and other mutants will be applied. Mutants, clearly producing fertile unreduced pollen in successive generations, will be selected for transcriptome analysis and gene identification.

Acknowledgements: We wish to thank Ortrun M. Scheid (GMI) for providing us the tetraploid *Arabidopsis thaliana* lines and Tim Bekaert (UGent) for setting up the Matlab script. This work is supported by FWO.

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