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Stress as a Trigger of Pollen Embryogenesis

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ABSTRACT The ability of microspores or young pollen grains (male gametophytes) to undergo developmetal switch to embryogenic (sporophytic) pathway exemplifies the concept of totipotency as applied to haploid postmeiotic cells. As a first step pollen is devoid of positional information provided *in situ* by the intact anther - by isolation and cultivation *in vitro* in artificial media. This is inevitably accompanied by some degree of stress response in microspore/pollen. It has been shown in both monocots and dicots that intentional stress treatment (mostly starvation or heat shock) greatly stimulates embryo induction rate. Using transgenic sHSP antisense *Nicotiana tabacum* we show that expression of small heat shock proteins is an integral part of successful embryo and later haploid plant production from pollen grains. Our recently published data show that sHSP chaperone function is optimal in the absence of ATP.

Key words : Heat shock proteins, pollen embryogenesis, stress

Abbreviations : HSP : heat shock proteins, sHSP : small heat shock protein. GUS : glucuronidase.

Introduction

The unintentionally discovered phenomenon of pollen embryogenesis (Guha and Maheshwari 1964) initiated explosion of studies aiming to use haploid plants produced in this way for breeding programs, as well as to understand cellular mechanism of the whole process in order to optimise the mostly empirical protocols for haploid plant induction. This technology contributed to successful breeding of new cultivars in many crop species in Europe, Asia and North America. However, the cellular mechanisms of the developmental switch from gametophytic to sporophytic pathway are still largely unknown and sometimes connected with rather wild hypotheses (Vicente et al. 1991; Bonet et al. 1998).

We have shown that the developmental transition to pollen embryogenesis is connected with changes in the nucleolus structure of the vegetative cell and cell cycle progression in tobacco pollen. The vegetative cell is normally arrested in G1 (G0) during the male gametophyte *in situ* development (Zarsky et al. 1990). However, upon transition to embryogenic development, after the starvation stress treatment, G1 arrest of the vegetative cell (embryo mother cell in this case) is released. Immediately after the transfer to the rich medium replication of DNA (S-phase) is initiated, concomitant with the onset of embryogenic developmental pathway (Zarsky et al. 1992). Although starvation stress has been used to induce pollen embryogenesis of tobacco mid-binucleate pollen grains (Kyo and Harada 1986), we have cloned a cDNA coding for a homologue of class I small heat shock proteins (sHSP), known to be induced by heat stress from starvation induced embryogenic microspores. Using heterologous HSP and 35S promoters linked to GUS reporter gene we have shown that general stress response is activated in mid-binucleate pollen during starvation period (Zarsky et al. 1995). This discovery, along with the well established practice of

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heat shock induced pollen embryogenesis in Brassica, has lead to the development of an optimised system combining both stresses (Touraev et al. 1996).

Here we will summarise shortly our new data related especially to pollen embryo induction rate in transgenic tobacco plant expressing antisense cDNA of sHSP. These plants suffer from inhibited accumulation of mRNAs coding for sHSP in response to stress treatment, and at the same time produce significantly less pollen embryos. In the discussion we will also include our recently published data on the regulation of sHSP chaperone function by ATP (Smykal et al. 2000).

Materials and Methods

Donor transgenic *Nicotiana tabacum* cv. Petit Havana SR1 plants were prepared in collaboration with the laboratory of prof. F. Schoffl (Tubingen, Germany). NtHSP18P cDNA (accession num. X70688) was cloned in antisense orientation under the heat stress inducible HSP (plants 2/5) or 35S promoters (plants 1/15, see also Discussion). Plants were cultivated in the greenhouse under the 16h light/ 8h dark regime at 25C. Heat shock response of individual plants was assessed using Northern and Western blotting with radioactively labelled sHSP cDNA or antibodies directed against sHSPs.

Microspores were isolated and induced to produce embryos *in vitro* essentially according to Touraev et al. (1996) . The only difference was that in the case reported here we have used heat stress (37C for three days) in the medium with 0.25 M sucrose. Induction of pollen embryogenesis was estimated microscopically after the DAPI nuclear staining using fluorescence microscopy. Results are displayed as the induction rate - proportion of embryo forming pollen grains from the whole pollen population.

Results and Discussion

It was firmly established that the 35S promoter, though regarded as a constitutive one, is not active during pollen development. We and others have shown that this promoter is activated at the onset of the embryogenic pathway in response to stress treatment of pollen.

Both populations of anti-sense tobacco transgenic plants (HSP or 35S promoter driven) display variable degrees of inhibition of accumulation of sHPS encoding mRNAs in response to heat stress as compared to the control (data not shown). The same holds true for the protein level investigated using anti-sHSP antibodies.

Our data on embryogenic response of the pollen populations isolated from control and transgenic antisense plants (Figure 1) show clearly reduced embryo production in both anti-sense plant populations - only about 50% of the control. Generally lower level of induction in this case relates to use of heat stress only with the elimination of starvation. Viability of the same populations studied using fluorescein-diacetate staining was not influenced - there were no significant differences between control and anti-sense plants. The same pollen populations were able to produce mature (starch filled) pollen to the same level as the control in the rich medium. This points again to the fact, that viability of pollen grains from antisense transgenes was not significantly affected. This data are in a good correspondence with the results published recently on Brassica by Smykal and Pechan (2000). Non responsive mutant of *Brassica napus* cv. Topas producing 50 times fewer embryos had substantially reduced expression of sHSPs in young pollen grains subjected to the androgenesis inductive heat stress. Comparison with pollen embryogenesis induced by colchicin treatment, which is also accompa-

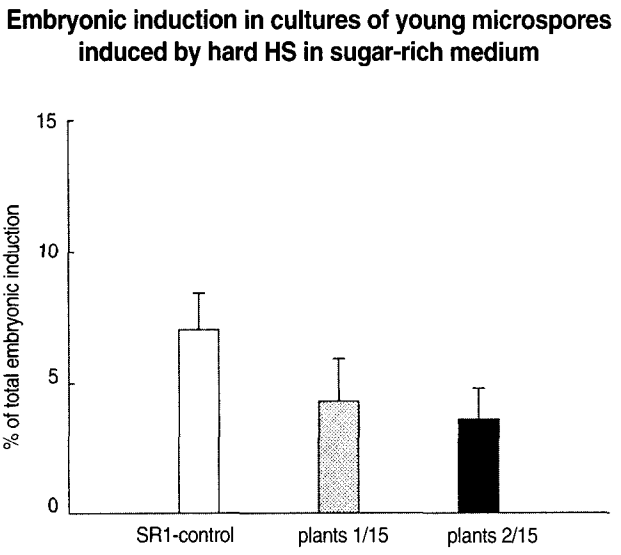

Figure 1. Microspore embryogenesis was induced and estimated as described in Materials and methods (three independent repetitions).

nied by sHSPs expression, has led authors to conclude that stress response including transcription of sHSPs, is one of the major events associated with changes in the pollen differentiation process that can lead to androgenesis (Smykal a Pechan 2000).

Looking for NtHSP18P chaperon function *in vitro*, we were surprised by our observation contradicting reports on ATP not interfering with sHSPs function (e.g. Lee et al. 1995). ATP not only inhibited sHSP chaperone function in concentration dependent manner but also inhibited the conformational switch imposed to this protein by increased temperature (Smykal et al. 2000). This means that at least some sHSPs in starving and or heat shocked pollen grains with low ATP content are active in the optimal cellular environment. We propose that during the recovery phase (with high ATP level reestablished), the protected and only partially denatured proteins are released from sHSP complexes and are refolded by HSP70 in ATP dependent manner.

In contrast to the hypothesis put forward by Bonnet at al. (1998), suggesting that pollen embryogenesis is an atavism towards a multicellular gametophyte, we prefer to understand it as an example of plant cell totipotency. Stress response here is a major dediferentiation mechanism clearing transcriptional as well as translational apparatuses from gametophytic information complexes and opening track for the default pathway towards cell division and embryo formation.

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