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Pistil Receptivity, Pollen Tube Growth and Gene Expression During Early Fruit Development in Sweet Pepper (*Capsicum annuum*)

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ABSTRACT

Glasshouse and laboratory studies were conducted to investigate stigma receptivity, pollen tube growth and identify genes associated with fertilization in sweet pepper (*Capsicum annuum* var. Golden Bell). Emasculated flowers were pollinated and growth of pollen tubes monitored. Tips of pollen tubes were observed in the ovary and ovules of sweet pepper within 36 and 48 h, respectively. Fertilization in sweet pepper, therefore, takes place two days after pollination. Pollen grains failed to germinate on the stigma of sweet pepper on the third day after anthesis, indicating a stigma receptivity of two days after flower opening. Analysis of cDNA clones corresponding to messages expressed after fertilization revealed a high level of expression of clones 1b06, 1e11, 1e12; 1f10, 2d09 and 2d05. Database comparisons showed that these cDNAs correspond to mRNA of genes associated with defense mechanisms, synthesis of cell wall structural protein, cell division and heat tolerance in plants.

Key Words: Gene expression; Pollen tube growth; Stigma receptivity; Sweet pepper

INTRODUCTION

Pollination is a major event in plant reproduction, which triggers fertilization, ovary growth and development. A number of biochemical and physiological changes, including changes in protein, sugar, starch, hormones and the rate of respiration take place in the style and ovary tissue after pollination (Gillaspy *et al*., 1993).

Most of these processes are mediated by programmed differential expression and regulation of gene. To understand the molecular regulation of these processes, the relevant genes differentially expressed during fruit development must be identified, cloned and studied. Recently, the molecular and genetic mechanisms by which pollination and fertilization trigger developmental pathways in the flower have been studied. Messenger RNAs expressed in early fruit development have been identified in crops such as tomato (Tieman & Handa, 1996) and peach (Callahan *et al*., 1993). Despite the fact that sweet pepper is a major horticultural crop, there is little or no information on the genes that are expressed after pollination and fertilization. The objectives of this study were to determine when fertilization occurs after pollination in sweet pepper and to isolate the genes that are specifically expressed during early fruit formation in sweet pepper.

MATERIALS AND METHODS

Three weeks old seedlings of sweet pepper variety 'Golden Bell' were transplanted into pots in a naturally lit glasshouse. Flowers were emasculated a day before anthesis and bagged to prevent natural pollination.

Pollen tube growth and pistil receptivity. To study pollen tube growth and pistil receptivity, flowers were emasculated and tagged to prevent chance pollination. On day 1, 2 and 3 after anthesis the stigmas of some emasculated and bagged flowers were pollinated by hand, after which flowers were re-bagged. The path and site of pollen tubes in the pistil were followed by staining Callose staining of pollen tubes was performed as described by Tang and Woodson (1996), and pollen tubes were visualized with a fluorescence microscope (E 800; Nikon, Tokyo, Japan). Pistil receptivity, taken as the germination of pollen on the stigma was also observed. Some emasculated flowers were also treated with naphthalene acetic acid (NAA), at a concentration on 10^{-5} M (determined in an earlier preliminary trial as the effective concentration for fruit setting), to study its effect on fruit growth. Bags covering pollinated and NAA treated flowers were removed three days after anthesis to allow for fruit development.

RNA extraction. Emasculated flowers were pollinated daily for three consecutive days after anthesis (DAA). Ovaries were harvested at 2 days after pollination (DAP) frozen in liquid nitrogen and stored at –80°C to be used for total RNA extraction. Total RNA was extracted as described by Dudareva *et al*. (1996).

Isolation of mRNA and suppression subtractive hybridization. Messenger RNA was isolated from total

RNAs of pollinated and non-pollinated ovaries using Oligo-dT30 < Super > (Takara Shizo Co. Ltd, Japan) and used for cDNA synthesis. The subtraction of non-pollinated ovary cDNA from pollinated ovary cDNA was done with suppression polymerase chain reaction (PCR)-based method (Clontech PCR-select cDNA subtraction kit, Clontech Laboratories, Palo, Alto, CA).

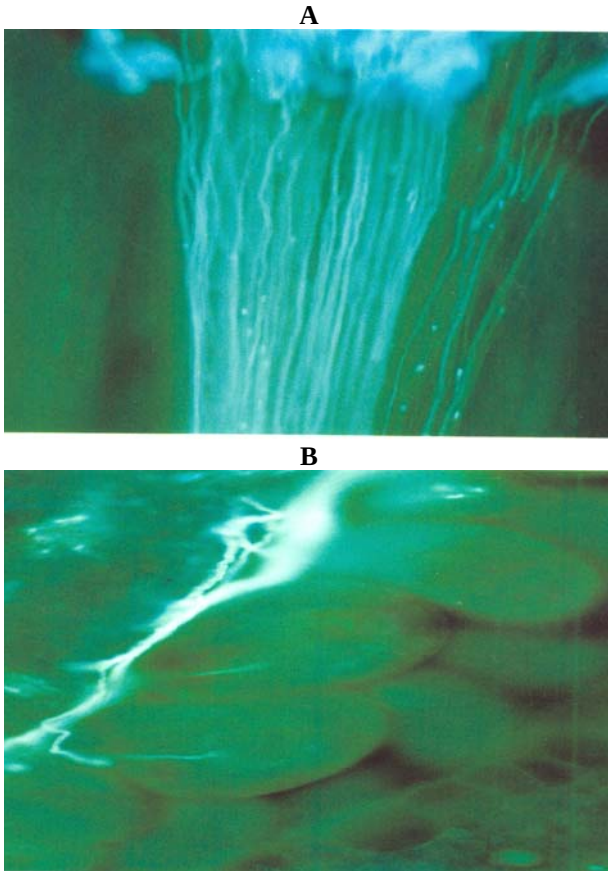
Cloning. The subtracted cDNA were inserted into a T/A cloning vector (pT 7 blue T-vector) using T/A cloning kit (Invitrogen) and then used to transform *E. coli*. Three hundred clones/colonies were picked at random and sequenced and genes identified by the Dragon Genomics Company (Yokkaichi, Mie-ken, Japan).

Macroarray analysis. Three hundred and eighty four clones selected at random were amplified by PCR using primers and the products spotted on nylon membranes. The membranes were then hybridized with 33 P-labelled probes prepared from 2-day un-pollinated and pollinated ovaries to compare the profile of genes expressed under both conditions.

RESULTS AND DISCUSSION

Fig. 1 shows the growth of pollen tubes through the style and ovule of sweet pepper flower. Our study revealed that stigmas of sweet pepper have a receptivity of three days. The pollen tubes reached the ovary 1.5 days after pollination but had not entered the ovules (data not shown). On the second day after pollination, pollen tubes were observed in the ovule. It is noteworthy that only one pollen tube was observed per ovule. The presence of pollen tubes in flower ovules 48 h after pollination suggests that fertilization in sweet pepper takes place from two days after pollination. Stigma of flowers retain their reception for a number of days after anthesis. The stigma in sweet pepper was found to be receptive (supported pollen grain germination) from 0 DAA to 2 DAA (data not shown). In Saw Palmetto (*Serenoa repens*), Carrington *et al.* (2003) observed stigma receptivity of 4 days. That is, depending on the species stigma receptivity may vary. Styles, stigmas and petals browned and withered 4 DAA. Fruit development in sweet pepper followed a simple sigmoid curve (Fig. 2) for both pollinated and naphthalene acetic acid treated flowers. Un-pollinated flowers aborted 5 days after anthesis. The ovaries of pollinated flowers were bigger though the rates of development of the ovaries were similar to ovaries treated with auxin Fig. 2. In sweet pepper (*Capsicum annuum*), as in most flowering species, the process of ovary development can be divided into three phases (Gillaspy *et al*., 1993). The earliest phase involves carpel initiation and ovary development up to anthesis, including the decision to proceed with fruit development if fertilization has successfully taken place. The second consists of a period of rapid cell division; whereas, the third is characterized by a marked cell expansion and by the final process of ripening (Gillapsy *et al*., 1993).

Fig. 1. Pollen tube growth in the style of Capsicum annuum var. Golden Bell flower a day after pollination (A) and into the ovules two days after pollination (B)



Treating emasculated flowers with naphthalene acetic acid stimulated fruit growth of sweet pepper and confirms the important role of auxin in ovary development and fruit growth (George *et al*., 1984). To identify genes induced by pollination or fertilization, cDNA library was constructed from poly (A^+) mRNA isolated from 2-day un-pollinated and pollinated ovaries. Subtracting cDNA of un-pollinated ovaries from those of pollinated ovaries allowed the identification of genes that might be involved in fruit set (Table I). In this study, 384 cDNA clones were randomly selected from the constructed cDNA library for sequencing. Genes that were observed to be highly abundant after macroarray analysis in the ovary of 2-day old pollinated flowers are listed it Table II. Analysis of sequenced genes showed that 74% of the total number of clones exhibited a homology to known genes including EST.

Over six candidate cDNAs were found with possible roles in pollination and sweet pepper. Eight ovule and stage specific genes have been characterized by Nadeau *et al* (1996). Clone 2e05 identified in this study has a high similarity to histone H3. Different classes of histones likely play important roles in the cell division phase of fruit growth and developmental regulation (Dong *et al*., 1998;

Fig. 2. Changes in weight of flower ovary during early development of *Capsicum annuum* var. Golden Bell. Un-planted (♦), naphthalene acetic acid treated (□) and pollinated (O) flowers

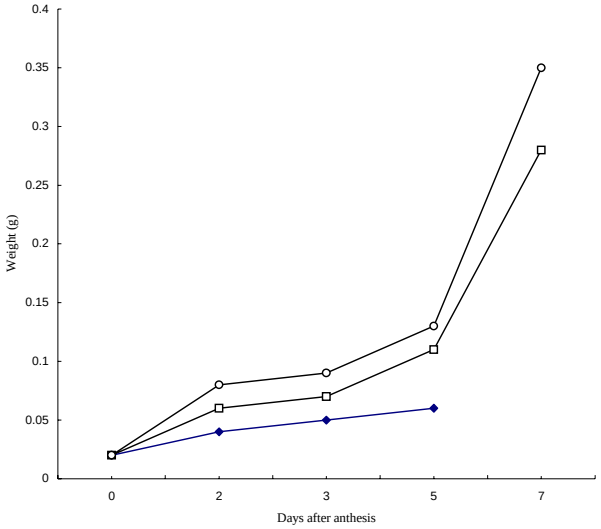
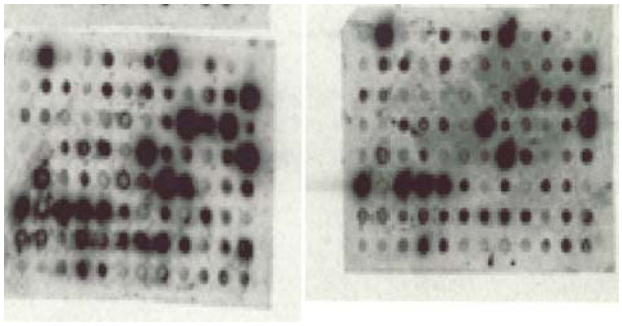


Fig. 3. Hybridization profiles corresponding to pollinated (A) and unpollinated (B) ovaries. Arrow indicates highly expressed genes in pollinated ovaries after macroarray analysis



Joubes *et al*., 1999). The high abundance of histone H3 observed at this early stage of fruit development (2 days after pollination) during, which there is high rate of cell division (Gillapsy *et al*., 1993) is a confirmation of the important role that histone H3 plays in the growth of fruits.

A glycine-rich RNA-binding protein, clone 1e11, was observed to be associated with pollination and fertilization in sweet pepper. Naqui *et al*. (1998) cloned a cDNA encoding a putative *Nicotiana glutinosa* glycine-rich RNA binding protein from TMV induced cDNA library and suggested its possible involvement in plant defense and possibly the development of flower ovaries into fruits. Glycine-rich RNA-binding proteins have also been implicated in many physiological processes, including ABA induction (Gomez *et al*., 1988), water and chemical stress tolerance (Gomez *et al*., 1988; Didierjean *et al*., 1992) and light and temperature-entrained circadian rhythm (Carpenter

Table I. Highly expressed genes after first macroarray analysis (first screening)

Clone No.	*Putatively identified gene*
1a04	Photosystem II CP 43 protein (Cercidiphyllum japonica)
1b0b; 3a04	UDP-galactose-4-epimerase (Cymopsis tetragonoloba
1e11	Glycine rich RNA-binding protein (Arabidopsis thaliana)
1e12	Putative senescence-associated protein (Pisum sativa)
1f10	Heat shock cognate protein (Lycopersicon esculentum)
1f12	Embryonic flower 2 (A. thaliana)
1g01	D 123-like protein (A. thaliana)
1g11	Remorin(Solanum tuberosum)
1g12	Cell wall invertase (Fragaria ananassa)
2c03	Glycine hydroxymethyltransferase-like protein(A thaliana)
2c08; 2c12	Putative protein (A. thaliana)
2c11	Chaperon 21 precursor (L. esculentum)
2e05	Histone H3 (Mastigomoeba balamuthi)
2f07	Hypothetical protein (A thaliana)

Table II. Genes highly expressed after macroarray analysis and their function

Clone No.	*Putatively identified gene*	*Function*
1b06; 3a04	UDP-galactose-epimerase (*Cymopsis tetragonoloba*)	Cell wall synthesis
1e11	Glycine rich RNA-binding protein (*Arabidopsis thaliana*)	Wall structural protein
1e12	Putative senescence-associated protein (*Pisum sativa*)	Senescence
1f10	Heat shock cognate protein (*Lycopersicon esculentum*)	Stress and defence related
1f12	Embryonic flower 2 (*A. thaliana*)	Repression of flowering
2e05	Histone H3 (*Mastigomoeba balamuth*)	Cell division

et al., 1994; Heintzen *et al*., 1994).

Clone 1b06, is a putative UDPG epimerase, whose main function in plants is the production of UDPD-galactose, which is utilized in the biosynthesis of low molecular weight galactosidases and cell wall polymers (Campbell & Reid, 1982). These low molecular weight compounds play an important role in cell wall formation. Clone 1b06 may therefore encode cell wall proteins required for the enlargement of sweet pepper.

Clone 2d09 encodes a chitinase that catalyse the hydrolysis of polysaccharides of many fungal cells and has been suggested to be involved in protecting plants against fungal pathogens (Boller, 1987). For fruits to develop and grow to maturity the role of this gene cannot be overemphasized.

Heat shock cognate protein 80, clone 1f10, identified in this study is a major heat-shock protein induced by heat and specific developmental events. (Lindquist & Craig, 1988; Nagao & Key, 1989). Its expression has been reported to be highest in flower ovaries and a single gene is responsible for its synthesis (Xiao & Lis, 1989). The expression of heat shock protein is vital for the growth and development of the fruits in under high temperature conditions. Such a gene could be introduced into pepper varieties developed for production in the tropics. Clone 1e12 encodes enzymes, pathogenesis related protein, cell wall and stress-response proteins (Juan *et al*., 2001) and may

therefore be very important regarding fruit growth and development.

CONCLUSION

In the present study, we succeeded in isolating various genes, which are expressed in the early development of sweet pepper. The information derived may be transferable to a wide range of crop species that share similar aspects of ovule and embryo sac development.

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