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**Biological Thinning of Fruit Set by RNAase in
Deciduous Fruit Trees**

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Biological thinning of fruit set by RNase in deciduous fruit trees.

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BARD-Final Scientific Report.

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ABSTRACT

Fruit thinning is a common and necessary practice for commercial fruit production in many deciduous tree fruit species. Fruit thinning in apple may be accomplished with a variety of chemical thinning agents, but the use of these chemicals is a subject of environmental concern. It has been shown recently that RNase enzyme, secreted from the stigma and the style, inhibits pollen germination and pollen tube elongation. In this study we have been able to show that *Aspergillus niger* B-1 RNase can effectively inhibit peach and apple pollen germination, and tube elongation in-vitro, as well as thin fruit in peach and apple, and reduce the number of seeds in citrus. The objectives of the research were to determine the conditions for effective thinning of (USA and Israel), develop fermentation process for cost effective production of RNase from *A. niger*.(Israel), and clone apple S-RNase cDNA (USA). All the objectives of the research were addressed. We have determined the optimal fermentation conditions for cost effective production of the *A. niger* at a 20,000 liters scale. The *A. niger* B1 RNase was isolated to homogeneity and its kinetic and biochemical properties including it's N-terminal sequence were fully characterized. The field test results both in Israel and California have shown variability in effectiveness and more work is needed to define the RNase concentration necessary to completely inhibit pollen development. Plant transformation vectors expressing anti-sense apple S-RNase genes were constructed (USA) with an attempt to produce self compatible transgenic apple trees. Bovine S-Protein cDNA was cloned and successfully expressed in E.coli (Israel). Plant transformation vector expressing the S-Protein gene was constructed (USA) with an attempt to produce transgenic plants expressing S-protein in the style. Exogenous application of S-peptide to these plants will result in active RNase and consequently prevention of fertilization.

OBJECTIVES

The objectives of the research (as modified after the review of the original proposal) were:

1. Determine the conditions (formula, timing etc.) for effective thinning of peach, nectarine and apple cultivars by *Aspergillus niger* RNase. (USA and Israel)
2. Determine the optimal fermentation conditions for cost effective production of RNase from *A. niger*.(Israel).
3. Isolate the *A. niger* B1 RNase and the apple stylar RNase determine its N- terminal sequence and prepare antibodies to the enzymes using either pure enzymes or conjugated peptides derived from the amino acid sequences (*A. niger* in Israel apple in USA).
4. Clone the apple cDNA clones and determine it's nucleotide sequence (USA).

Objective 4 was modified since the genes were already cloned and sequenced. Therefore, plant transformation vectors expressing anti-sense apple S-RNase genes were constructed (USA) with an attempt to produce self compatible transgenic apple trees.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

ISRAEL

The results of the Israeli team is presented in two published papers (see appendix), one issued US patent, and the following manuscript. The papers and the patent are enclosed.

Characterization of *Aspergillus niger* B-1 RNase and its applications in thinning fruit set of deciduous fruit trees and seedless production in citrus fruits.

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Abstract

Aspergillus niger B-1 RNase was purified to homogeneity. It was found to contain two isoforms of 32- and 40- kDa glycoproteins, sharing a 29-kDa protein moiety. The optimal RNase activity was observed at a temperature of 60°C and pH 3.5. The enzyme inhibited peach pollen germination and tube growth *in vitro* as well as *in vivo*. In field experiments, the RNase caused a reduction in reduced fruit set and interfered with the embryo development. The biological effect of the RNase may be of horticultural value, due to its potential as an environmentally safe biological thinning agent for stone fruits.

Introduction

In self-incompatible plants, the growth of self-pollen is arrested in the stigma or style, before fertilization occurs. Self-incompatibility is often controlled by a single multiallelic S-gene¹. A common outbreeding mechanism is gametophytic self-incompatibility, in which a pollen tube bearing an S-allele identical to one of the two alleles of the pistil fails to achieve fertilization²⁻⁶. In members of Solanaceae and Rosaceae, the S-alleles have been found to encode stylar-specific S-glycoproteins exhibiting ribonuclease (RNase) activity, as well as the ability to inhibit the growth of self-pollen tubes⁷⁻¹¹. Two domains in the primary structure of these S-glycoproteins, containing one histidine residue each,

show strong homology to *Aspergillus oryzae* RNase T₂, *Rhizopus*
niveus RNase Rh⁸, and *A. saitoi* RNase M¹².

In some plants pistilar non-S-allele-specific ribonucleases were found to be produced. For example, RNase X has been reported to be shared by both self-incompatible *Nicotiana glauca* and self-compatible *N. glauca*⁸. In self-incompatible *Petunia inflata* RNase X2 shares homology with S-RNases, yet its gene is not located on the S-locus¹³. The biological traits and function of these RNases were not fully determined yet. In self-compatible peach (*Prunus persica*) and calamondin (*Citrus reticulata*), the stigmatic diffusate contains RNase activity. These stigmatic RNases inhibit pollen germination and tube elongation *in vitro* in a dose-responsive manner. In these two species *in vitro* studies suggest that nonspecific RNases play a role in the pollination process^{14,15}.

Aspergilli have been reported to produce extracellular RNases¹⁶. *A. niger* is also used as a source for important substances and enzymes in the food industry, such as citric acid and pectinases¹⁷⁻¹⁹.

The effect of nonspecific RNase may be of horticultural value: in many deciduous fruit trees, yields of commercial value depend upon massive thinning of the flowers and fruitlets. In stone fruits (peach,

plum ect.) the thinning process is currently performed manually. In the present work we describe the production and purification of *A. niger* B-1 extracellular RNase. Its effect on pollen tube growth *in vitro* and *in vivo* was studied, together with its relation to embryo development and fruit production.

Results

Purification and characterization of *A. niger* B-1 RNase

A. niger grown in liquid culture produced considerable amounts of extracellular RNase. A temperature of 60°C and pH 3.5 were found optimal for RNase activity, and were adopted as the standard conditions for RNase assays.

The purification of RNase consisted of three steps (Table 1). The crude filtrate contained 0.05 mg/ml protein, the RNase activity of which was 1000 units/ml. The pooled fractions around the active RNase peak eluting at 0.62 M NaCl (Fig.1a) from an EMD-TMAE column, contained 0.1 mg/ml protein, with an RNase activity of 40,000 units/ml. In the final step, the RNase was eluted from a Mono Q column at 0.5 M NaCl (Fig. 1b); the eluant had protein concentration and RNase activity of 1.05 μ g/ml and 543,000 units/ml, respectively. Two major protein bands, of 40 and 32 kDa, were observed following SDS-PAGE of the

purified RNase B-1 (Fig. 2). An activity gel showed RNase activity to be restricted to both proteinous bands. When subjected to PNGase F, a single protein band appeared at 29 kDa. RNase activity was retained after PNGase digestion (not shown).

The effect of RNase on pollen tubes and fruit production

In vitro, 75% of the control pollen grains germinated and the pollen tubes reached about 0.5 mm in length. Addition of *A. niger* B-1 RNase to the growth medium reduced the percentage of germination and the length of the pollen tubes, in a dose responsive manner (Fig. 3). Here, RNase had an impressive reductive effect, since converting RNase activity to protein concentration showed that 50 units/ml, representing 0.1 mg/ml protein, were found lethal. For comparison, 125 mg/ml of BSA reduced only half of pollen germinability and tube growth (not shown).

In vivo, control pollen tubes of peach were observed growing through the stigmatic tissue and directed into the style, 48 h after pollination (Fig. 4a). The same result was obtained in styles treated with buffer alone. In contrast, pollen grains germinated on stigmas treated with *A. niger* B-1 RNase, produced short pollen tubes, which appeared to lack any growth orientation, and failed to penetrate the stylar tissue (Fig. 4b). In tangerine only a small portion of the stigmatic tissue, of

which the diameter is 2-3 mm, could be observed in the microscopic field. Therefore, only few pollen tubes were captured in the microscopic field, as shown in Fig. 5. However, the difference between the normal growth of the control pollen tubes (Fig. 5a) and the irregular growth of the RNase-treated pollen tubes (Fig. 5b), was clearly observed.

In nectarine cv. Fantasia, *A. niger* B-1 RNase caused a reduction in fruit set (Table 2). In branches that were remained untreated or sprayed with buffer with triton X-100, 48.3% and 36.3% fruits, respectively were obtained. On the other hand, ranches treated with 500 and 1000 units/ml of RNase set 23.3% and 18.4% fruits, respectively, indicating a dose dependent response. The reductive effect of the buffer itself might be a consequence of its low pH (needed to maintain RNase activity), which may hurt the flowers. However, the excessive thinning effect was clearly related to the RNase. Viability tests showed that in the control (flowers untreated or spayed with buffer) the embryos were stained red, (Fig. 6a), whereas the cut surface of embryos born in RNase-treated flowers was unevenly stained, showing brown necrotic tissues (Fig. 6b).

Discussion

An effective fermentation and purification process was developed for the production of *A. niger* B-1 RNase²⁰. A temperature of 60°C and a pH of 3.5, values which were optimal for *A. niger* B-1 RNase activity, are also optimal for the activity of β -glucosidase produced by this strain²¹. In related species, extracellular RNases have been found to require similar conditions for optimal activity. For example, *A. niger* NCR-A-1-233 RNase (50°C, pH 3.5), RNase M (50°C, pH 4) RNase T₂ (pH 4.5), RNase Rh (50°C, pH 5) and RNases from *A. fonssecaeus* and *A. flavus* (pH 5)^{22,23}. All of these RNases have molecular masses between 24-34 kDa. They are also base-nonspecific enzymes with a preference to adenilic acid^{12,24}.

The secretion of considerable amounts of RNase should have a substantial biological role. In microorganisms, extracellular RNases are generally accepted to contribute to the digestion of polyribonucleotides present in the growth medium, thereby giving rise to diffusible nutrients¹⁶. These so-called nutritive characteristics of RNases have also been described in plants: cells of tomato suspension cultures secreted RNase into the growth medium in response to phosphate starvation²⁵. Nevertheless, more research is needed for a complete understanding of the physiological role of fungal extracellular RNases.

A. niger B-1 RNase was purified to a high degree of homogeneity. PNGase deglycosylation followed by the appearance of a single 29-kDa band indicates heteroglycosylation of this protein. RNase T₂²⁶ and RNase M^{12,27} have also been found to contain different isoforms, sharing the same protein moiety, but differing in carbohydrate content. In RNase T₂ the occurrence of different-size glycan chains has been suggested to be due to enzymatic degradation by glycosidases, which are also present in *A. oryzae* medium-filtrate²⁶. Carbohydrates are considered to play a role in the maintenance of glycoprotein stability and in improving their solubility²⁸. In *Petunia hybrida*, four S-RNases were found to contain one to three N-linked glycan chains each. Deglycosylation did not reduce RNase activity, suggesting that the carbohydrate chains are involved in the self-recognition reaction on the pollen tube surface²⁹. In *N. alata*, the glycan chains attached to the S-RNases were assumed to influence their three-dimensional structure, thereby playing a role in their allelic specificity³⁰. However, in *P. inflata* S₃ protein, which normally has a single glycan chain, was mutagenized and gave rise to S₃(N29D), a nonglycosylated protein. The ability of S₃(N29D) protein to gain complete inhibition of S₃ pollen

indicates that the carbohydrate moiety is not necessarily required for the self-incompatibility reaction³¹

In this work, *A. niger* B-1 RNase had a clear inhibitory effect on pollen germination and tube growth, *in vitro* as well as *in vivo*. Furthermore, in both peach and tangerine the appearance of the RNase-treated pollen tubes resembled the morphological features described in incompatible pollen tubes for *Petunia hybrida*³² and *Papaver rhoeas*³³. The pollen tubes were short, burst easily, had irregularities in wall deposition, and lacked a definite growth orientation. In gametophytic self-incompatible plants, the S-RNases have been found to share homology with RNase T₂, RNase Rh and RNase M^{3,8}. *A. niger* B-1 RNase pollen-growth inhibition appears to be similar to that of S-RNases, despite its nonspecificity.

In nectarine RNase treatment reduces fruit set, indicating that RNase, by inhibiting pollen tube growth in the style, may lead to a decrease in the fertilization rate. During the first month following blooming period, fruitlets born on RNase-treated branches were smaller and showed larger falling ratio than in the control²⁵. The fact that RNase affected the embryos viability, may indicating its potential to interfere with the fertilization process.

In conclusion, *A. niger* B-1 was found to be an efficient source of RNase. The *A. niger* B-1 RNase inhibited pollen germination and tube growth nonspecifically, but the treated pollen tubes displayed morphological traits similar to those in gametophytic self-incompatibility. Of course, much work need to be devoted to form the RNase into a horticultural product. However, based on this work the potential of its biological effect is clear, with its additional advantage of being an environmentally safe agent.

Experimental protocol

Preparation and purification of *A. niger* RNase. *Aspergillus niger* was grown in liquid culture containing 1% (w/v) wheat flour and 0.05% (w/v) ammonium sulfate. The mixture was brought to pH 3.5 with HCl and autoclaved. An inoculum of about 10^6 spores was suspended in 100 ml of medium and incubated at 30°C in an orbital shaker, at 200 rpm for 7 days. The growth medium was passed through a 0.2- μ m membrane and dialyzed three times against 10 volumes of 2 mM sodium acetate pH 6. Two liters of dialyzed solution were loaded onto a Fractogel EMD-TMAE 650 (M) 26/10 (Merck) column, equilibrated with 20 mM sodium acetate pH 6. Bound proteins were eluted with a 500-ml linear gradient of 0-1 M NaCl in the same buffer,

using a fast protein liquid chromatography (FPLC) system (Pharmacia) at a flow rate of 5 ml/min. The fractions exhibiting the highest RNase activity were pooled and dialyzed against 2 mM sodium acetate pH 6, and a 50-ml aliquot was loaded onto a Mono Q 5/5 HR (Pharmacia) column, equilibrated with 20 mM sodium acetate pH 6. The elution was performed as with the EMD-TMAE column, except that only 10 ml of a 0-1 M salt gradient were used, at a flow rate of 1 ml/min. Proteins were monitored at 280 nm and measured according to Bradford³⁴ using BSA as a standard. Different fractions were analyzed by 12.5% SDS-PAGE³⁵. An RNase activity gel was run according to Roiz & Shoseyov¹⁴.

The purified RNase was enzymatically deglycosylated according to the procedure described by Broothaerts et al.²⁹. The enzyme was mixed with 0.5% (w/v) SDS and 5% (w/v) β -mercapthoethanol and heated at 100°C for 5 min. Once cooled, the reaction mixture was diluted 2.5-fold with buffer containing 50 mM sodium phosphate pH 7.5, 25 mM EDTA, 1% (w/v) Triton X-100 and 0.02% (w/v) sodium azide. Peptide-N-glycosidase F (PNGase F, Boehringer-Mannheim) was added to a final concentration of 20 units/ml and incubation was performed overnight at 37°C. The sample was then mixed with sample application buffer, heated at 100°C for 5 min, and analyzed by 12.5% SDS-PAGE.

RNase assays. The optimal conditions for RNase activity were determined in a range of temperatures, from 20-100°C and pHs from 2.5-7. The RNase activity assay was modified from Brown and Ho³⁶. samples of 10 μ l were added each to 490 μ l of ice-cold 20 mM sodium acetate at one of the pHs examined, containing 4 mg/ml yeast RNA (Sigma). Half of the mixture was immediately moved to another tube containing 50 μ l of 0.75% (w/v) uranyl sulfate in 25% (w/v) perchloric acid (stop mixture), for use as a blank. The rest was incubated for 10 min at one of the temperatures being tested, briefly cooled on ice and 50 μ l of stop mixture were added. Following centrifugation at 15,000g for 5 min, the supernatant was diluted 20-fold with distilled water and the absorbance was determined at 260 nm. One unit of RNase activity was determined as the amount of enzyme releasing soluble nucleotides at a rate of one A 260 nm unit per min.

The effect of *A. niger* B-1 RNase on pollen tube growth

Peach pollen was germinated *in vitro* in liquid culture, as described by Roiz and Shoseyov¹⁴. Pollen grains were suspended in aliquots containing 100 μ l of 15% (w/v) sucrose, 100 μ g/ml boric acid, 200 μ g/ml magnesium sulfate, 200 μ g/ml calcium nitrate and different

concentrations of *A. niger* B-1 RNase. After incubation overnight at 25°C in a dark chamber, percent of germination was recorded. Pollen tube length was examined with an eyepiece micrometer.

The effect of RNase treatment on pollen tube growth tested was also *in vivo*. In peach cv. Almog and in tangerine (*Citrus reticulata*, Blanco) cv. Murcott intact flowers were sprayed with 100 units/ml of *A. niger* B-1 RNase in 20 mM citrate buffer pH 3.5 at the very beginning of anthesis. In each species additional flowers at the same stage were sprayed with buffer alone, or remained untreated, as controls. After exposure to open pollination for 48 h, the styles were fixed in 3:1 acetic acid : ethanol for 24 h, washed with distilled water and imbibed overnight in 8 M sodium hydroxide. After thoroughly washing in distilled water, the styles were cut longitudinally, immersed in a drop of 0.1% (w/v) aniline blue in 0.1 M potassium phosphate on a slide and carefully squashed with a cover glass. Pollen tubes were observed by epifluorescence microscopy (Olympus BX40).

The effect of B-1 RNase on fruit production

In nectarine cv. Fantasia in Rosh Zurim, branches of 30-40 cm long, bearing approximately 10% open flowers, were sprayed with different concentrations of RNase in 20 mM citrate buffer pH 3.5 and 0.025% triton-X 100. Untreated branches, and branches sprayed with only

buffer with triton-X 100, served as controls. The branches were sprayed at 2- to 3-days intervals during spring 1994. A month after cessation of the blooming period, young fruitlets started to fall. At that period, their number per branch and their weight were examined in each treatment. For viability test, seeds were cut longitudinally through the embryo and immersed in 1 % 2,3,5-Triphenyl tetrazolium chlorid in water for 4 h at 20°C in a dark room. Viable tissues were indicated by a red staining.

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Purification Step	Total units	Protein concentration (mg/ml)	Recovery (%)	Specific activity (units/mg protein)
Crude filtrate	2,000,000	0.05	100	2,000
EMD-TMAE	1,120,000	0.1	56	400,000
Mono Q	652,000	1.05	32.6	517,143

Table 1. Purification of RNase from growth medium filtrate of *A. niger* B-1. Protein concentration and RNase activity were monitored during enzyme purification.

Treatment	Flowers (total number)	Fruit set (%)
Untreated	169	48.3 a
Buffer	143	36.3 b
500 units/ml RNase	148	23.3 bc
1000 units/ml RNase	106	18.4 c

Table 2. Fruit set in nectarine cv. Fantasia in Rosh Zurim. The buffer was sodium citrate pH 3.5, with 0.025% triton X-100. The same buffer was used for the RNase solutions. Fruit set is expressed as the percentage of fruits per flowers. Each result represents the average of five measurements. Values followed by different letters are significantly different ($P \leq 0.05$).

Figure 1. Anion exchange chromatography of *A. Niger* B-1 RNase. The solid line represents absorbance at 280 nm and the dashed line represents RNase activity. a. The crude filtrate was dialysed and loaded onto EMD TMAE column. b. The pooled fractions collected around 0.62 M NaCl at step a, were loaded onto Mono Q column.

Figure 2. SDS-PAGE zymogram of the *A. niger* B-1 RNase purification steps. Lane 1. Crude filtrate. Lane 2. Proteins eluted from the EMD-TMAE column at 0.62 M NaCl. Lane 3. Proteins eluted from the Mono Q column at 0.5 M NaOH. Lane 4. As in lane 3, except that the proteins were assayed for RNase activity by diffusion plate assay¹³. Lane 5. Purified RNase, after deglycosylation by PNGase F. Lanes 1-3 and 5 are stained with coomassie blue.

Figure 3. The effect of different concentrations of *A. niger* B-1 RNase on peach pollen germination (solid line, black squares) and pollen tube length (dashed line, empty squares) *in vitro*. For the percentage of germination at least 100 pollen grains in each sample were monitored. For pollen tube length 10 pollen tubes in each sample were measured. Each point represents the mean of three replicates.

Figure 4. The effect of *A. niger* B-1 RNase on peach pollen tube growth in the stigma and the upper part of the style. a. A control flower, 48 after exposing to open pollination. b. A flower that was treated with RNase before pollination. Scale bar = 0.2 mm.

Figure 5. The effect of *A. niger* B-1 RNase on tangerine pollen tube growth in the stigma. a. A control flower, 48 h after exposing to open. b. A flower that was treated with RNase before pollination. Scale bar = 0.02 mm.

Figure 6. Viability tests: Nectarin cv Fantasia seeds longitudinal cut surfaces, that were stained with 2,3,5-Triphenyl tetrazolium chlorid. a. control. b. A seed born in an RNase-treated flower. Scale bar = 0.5 cm.

USA

Abhaya M. Dandekar, Henry J. Fisk and Sandra L. Uratsu

Figures 1, 3-9, 11-21 are in the appendix.

1. Isolation of S-RNase from apple:

The first strategy that we investigated was the isolation of S-RNase from the apple genome. We used two primers shown below that we obtained from the Shoseyov laboratory. These are S-allele specific degenerate primers.

The idea was to PCR clone the product and search an apple genomic library for the gene encoding the S-allele. For the latter we were able to make a good apple genomic library. This was made using DNA obtained from the Golden Delicious cultivar 'Greensleeves' in the cosmid vector 'SuperCos 1'. The primary library contained 1.7×10^6 cfu with a mean insert size of about 35-41kb. A good portion of this primary library representing 4.5×10^5 recombinants was amplified and distributed into 10 lots (containing about 2×10^9 cfu/ml). We have estimated that this amplification contains several genome equivalents and have confirmed this using a known apple gene. We have used the apple Sorbitol-6-phosphate dehydrogenase sequences and made primers and have been

able to amplify the corresponding genomic sequences from 2 of the 9 lots tested. We were also successful in cloning the this gene from one of these lots. The PCR amplification using the S-allele specific degenerate primers with apple genomic DNA did not result in the amplification of the S-allele. Several of the PCR products were cloned and sequenced and compared to known sequences without any success.

The alternative approach to clone S-allele from apple is through stylar cDNA libraries. This was not pursued as this work was already being done in Japan by Dr. Hidenori Sassa (Sassa et al., 1994; 1996) and in Belgium by Dr. Wim Broothaerts (Broothaerts et al., 1995). We are in contact with both Dr. Sassa and Dr. Broothaerts both of whom have now cloned stylar specific RNases from apple. Dr. Broothaerts has agreed to collaborate with us and has sent us cDNA corresponding to 2 stylar specific RNase that have been shown to display incompatibility reactions in Fuji and Royal Gala apples discussed below. Our approach is to express the antisense in a tissue specific manner in the styles of apple flowers. In order to accomplish this we needed a stylar specific promoter from some other plant since no such promoter has as yet been isolated from apple. We obtained from Dr. Chuck Gasser (U.C. Davis) a promoter of a novel chitinase gene (ChiP, Gasser et al., 1989) expressed at high levels in stylar transmitting tissue of tomato. Although this was used to successfully express SRNase in tobacco (Murfet et al., 1994) more recent work suggests that the CaMV35S promoter gives a more

effective antisense suppression of self-incompatibility in *Nicotiana* (Murfett et al., 1995). Therefore, in addition we have used as a comparison the UBQ10 promoter from *Arabidopsis thaliana* expressed generally in all tissues like the CaMV35S promoter. The UBQ10 promoter was isolated by Dr Judy Callis (U.C. Davis) from one of the ubiquitin genes of *Arabidopsis* (Callis et al.1995).

2. Construction of vectors for the expression of RNase:

S-Protein RNase: A plasmid pBS-Sprot (Fig. 1) was obtained from the Shoseyov laboratory. This was a pBluescript plasmid with a 370 bp insert containing the structural sequence encoding the S-protein. Since the sequence of this insert was determined, 3 primers (Fig. 2) were designed for insertion into our Ubi3 promoter vector pDU95.2103 (Fig. 3), one for the 5' end (SPROT-1) and two for the three prime end (SPROT-1 and SPROT-3). Restriction sites were included in the primer sequence to facilitate the cloning of the amplified fragment.

Fig. 2. Primers for the amplification of S-protein coding region.

SPROT-1

BamHI
 5' ⁶⁵ GGGATCCATGGGTA ACTATTGCAACCAGATGATGAAGA ⁹⁵
 3'.....SPROT-1

SPROT-2

³⁴⁰ ³⁷⁰ BglII
 3' GGGCATGCACGGTCACGTGAAGCTGCGAACTAA GATCT
 5'..SPROT-2

SPROT-3

BglII

340 370
 3' GGGCATGCACGGTCACGTGAAGCTGCGAACTTCTAGAG
 5'..SPROT-3

We decided to express the S-Prot coding region as a translation fusion with

ubiquitin sequences of the ribosomal protein gene (ubi3) from potato as previously described by Garbarino and Belknap (1994). This gene gives expression in all tissues especially meristematic tissues. Expression can be obtained as either a transcriptional or translational fusion with ubi3 (Garbarino and Belknap, 1994). In the case of the latter the fusion is made in-frame with the ubiquitin coding sequences downstream from the natural cleavage site. The * designates where cleavage with the ubiquitinated sequences occurs in the fused protein. The in-frame fusion with the ubiquitin sequences is accomplished at the BamHI/SmaI site in the vector which puts the N-terminal of the fused protein one amino acid downstream

from the natural cleavage site of the ubiquitin protein sequence (designated

by *, see below).

Ubi3

BamHI SmaI

pDU96.2601
 5' CTC CGT GGT GGT GGA TCCCCGGGAATTCCC 3'
 3' **GAG GCA CCA CCA CCT AGGGGCCCTTAAGGG** 5'
 leu arg gly gly*gly

L R G G * G

PCR amplification was carried out with SPROT-1 in combination with SPROT-3. The PCR products were then cloned into pCR2.1 (Invitrogen, San Diego CA) cloning vector obtaining pTA-Sprot (Fig. 4) that contained the desired PCR product. The plasmid pTA-Sprot was then digested with BamHI and BglII to remove the coding region of S-protein and then cloned into pDU96.2601 (Fig.3) digested with BamHI. The transformants were screened to obtain pDU96.2914 that contained the Sprot structural sequence in the correct orientation with respect to the Ubi3 promoter (Fig. 5). The plasmid pDU96.2914 was then digested with NotI and ligated into the binary plasmid vector pDU95.1001 (Fig. 6) digested with the same enzyme to obtain pDU96.3326 (Fig. 7). Restriction analysis followed by Southern blot analysis was used to confirm the map of pDU96.3326 (Fig. 7).

The binary vector pDU96.3326 (Fig. 7) was introduced into EHA101 a disarmed strain of *Agrobacterium tumefaciens*. Currently transformation experiments with tobacco cv. SR1 are in progress to obtain transgenic tobacco expressing the S-protein.

S-allele RNase: Almost 10 different S-alleles have been cloned from apple (Broothaerts personal communication). We focused upon the major S-alleles of the apple varieties Gala and Fuji. The S-alleles for Gala have been identified as S2 and S5, whereas, for Fuji they are S1 and S9. Since the DNA sequence homology of these genes are very

closely related the expression of one S-allele should be sufficient for inactivating both. In the case of Gala (S2,S5) we used S2 and for Fuji (S9, S1) we used A1 which is 93% identical to S1. The DNA sequences for A1 (Fig. 8) and S2 (Fig. 9) and the plasmids containing these two genes were obtained from Dr. Broothaerts.

Fig. 10. Primers for the amplification of S-allele coding region.

Primers for the amplification of A1

5' GGGTACCATGGGGACCGGGATGATATATATG 3'

5' GGGTACCTTAATACTGAATATTGGTGGGGCA 3'

Primers for the amplification of S2

5' GGGTACCATGGGGACTACGCGGATGGTATAT 3'

5' GGGTACCTTAATACAGAATATGATTGGTGGG 3'

The A1 gene was amplified using the above (Fig. 10) primers using the plasmid containing the cloned A1 gene as template. The PCR product was cloned into pCR2.1 obtaining pCRA1 (Fig. 11). Similarly the S2 gene was amplified using the above two primers using the plasmid containing the cloned S2 gene as template. The PCR product was cloned into pCR2.1 obtaining pCRS2 (Fig. 12). These primers contain KpnI sites on the 5' ends, thus the PCR products can be excised with KpnI. We decided to use two promoter sequences to express these S-alleles, the first was the ChiP promoter obtained from Dr. Gasser (U.C. Davis). The other was the UBQ10 promoter obtained from Dr. Callis (U.C.

Davis) and is the promoter for one of the ubiquitin genes isolated earlier from *Arabidopsis* (Callis et al, 1995). A plasmid pHK7 (Fig. 13) was obtained from Dr. Gasser that contained the ChiP promoter. This plasmid was modified to facilitate the construction of gene cassettes using the S2 and A1 alleles. The plasmid pHK7 was digested with NcoI and KpnI to remove the GUS coding region and this fragment was replaced with the following synthetic oligonucleotide to restore the NcoI and KpnI sites and to introduce an EcoRI site. The new plasmid was designated pCHiP (Fig. 13).

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      5'      CGAATTCC      3'
      3'      CATGGCTTAAGGGTAC      5'

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We obtained the promoter region for UBQ10 one of the *Arabidopsis* ubiquitin genes on a plasmid p6056 from Dr. Callis. The plasmid p6056 was digested with HindIII and BclI and the fragment containing the UBQ10 promoter was removed and ligated into pHK7 digested with HindIII and BglII. The plasmid pDU96.1501 was obtained that contained the UBQ10 promoter (Fig. 14). The plasmid pDU96.1501 (Fig. 14) containing the UBQ10 promoter was then digested with NcoI and KpnI to remove the GUS coding region and this fragment was replaced with the synthetic oligonucleotide shown above creating the plasmid pUB10P (Fig. 14).

Construction of gene cassettes for A1: The A1 region was removed from pCRA1 (Fig. 11) by digestion with KpnI and ligated into pCHiP (Fig. 13). Two plasmids were obtained that contained A1 in the sense (pCHA1.28, Fig. 15) and antisense (pCHA1.26, Fig. 15) orientations. Similarly, the A1 region digested as a KpnI fragment from pCRA1 (Fig. 11) was cloned into the KpnI site in the UBQ10 vector pUB10P (Fig. 14). Two plasmids were obtained that contained A1 in the sense (pUBA1.28, Fig. 16) and antisense (pUBA1.16, Fig. 16) orientations with respect to the UBQ10 promoter.

Construction of gene cassettes for S2: The S2 region was removed from pCRS2 (Fig. 12) by digestion with KpnI and ligated into pCHiP (Fig. 13). Only one plasmid was obtained that contained S2 in the sense (pCHS2.11, Fig. 17). No plasmids containing the antisense orientation were obtained. Similarly, the S2 region digested as a KpnI fragment from pCRS2 (Fig. 12) was cloned into the KpnI site in the UBQ10 vector pUB10P (Fig. 14). Again, only one plasmid was obtained that contained S2 in the sense (pUBS2.42, Fig. 18) orientation with respect to the UBQ10 promoter. No plasmids with the antisense orientations were obtained in spite of extensive screening.

Construction of binary plasmids: The chimeric gene cassettes present in the six constructs; pCHA1.28, pCHA1.26 (Fig. 15),

pUBA1.28, pUBA1.16 (Fig. 16), pCHS2.11 (Fig. 17) and pUBS2.42 (Fig. 18) were digested with NotI to remove the gene cassette and were ligated into pDU95.1001 (Fig. 6). Instead of obtaining the six respective binary vectors with each of the 6 gene cassettes we were only able to obtain 3; p01UBA1 contains A1 regulated by UBQ10 in the sense orientation (Fig. 19), p01UB1A that contains A1 in the antisense orientation with respect to the UBQ10 promoter (Fig. 20) and p01UBS2 contains S2 in the sense orientation with respect to the UBQ10 promoter (Fig. 21). The remainder three binary could not be obtained in spite of several tries. Restriction analysis followed by Southern blot analysis was used to confirm the map of these three binary vectors.

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Determine the conditions (formula, timing etc.) for effective thinning of peach, nectarine and apple cultivars by *Aspergillus niger* RNase.

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RESULTS AND DISCUSSION

Effect of RNAase applications on fruit set in apple. Our 1996 data suggest that 5000 units/ml RNAase marginally increased fruit drop in 'Golden Delicious' apple (106 vs. 85 fruit per 100 clusters, Table 2) and stimulated somewhat greater fruit drop in 'Granny Smith' (Table 2). The lack of a response to 2000 units/ml in 1995, and the annual variation in the natural fruit set percentage (Table 3) suggest that further experimentation might utilize RNase concentrations in excess of 5000 units/ml, and several more years of data are needed to demonstrate convincingly and reproducibly that RNase has potential as a commercial fruit thinning agent. The need for further experimentation is underscored by comparing the 1995 peach results with those of 1996. In 1995, our data suggested that RNase applied at 2000 units per ml reduced fruit set by about 40% in 'Snoflake' and by about 20% in

'O'Henry' (see 1995 annual report). In 1996, even 5000 units/ml RNase did not reduce fruit set in peach relative to the control (Table 2).

Effect of RNAase on in vitro pollen germination and pollen tube elongation in apple and peach.

A. Pollen germination and tube elongation in vitro were affected similarly by *A. niger* RNase and bovine pancreas RNAase (Tables 4 and 5). At 1000 units/ml the bovine pancreas RNAase inhibition of 'Golden Delicious' pollen germination and tube growth was slightly greater than the inhibition by *A. niger* RNase.

There seemed to be a subtle stimulation of 'Golden Delicious' pollen germination and tube elongation by *A. niger* RNase at 10-100 units/ml. This phenomenon was less conspicuous in peach (Table 5). The point of particular note, however, is the lack of complete inhibition of pollen germination and tube elongation when exposed to 1000 units/ml *A.*

niger RNase. In 2 of the 3 'Golden Delicious' replicates, pollen germination persisted to a level of 80% of the control (Table 4), and pollen tube elongation averaged >60% of the control despite inclusion of 1000 units/ml in the liquid medium. Peach germination and tube growth in vitro appeared to be a little more sensitive (Table 5) than

apple (Table 4) at the two higher concentrations of RNAase (i.e., 100 and 1000 units per ml), but, there was still significant pollen germination and tube elongation at the highest RNAase levels employed. There was some suggestion that 'O'Henry' peach pollen was more sensitive than 'Snoflake' peach pollen at 1000 units/ml (Table 5).

In conclusion, on the basis of our data the biological effectiveness of RNase as a fruit thinning agent has not been demonstrated convincingly under California conditions even when tested on selected (vulnerable) subpopulations of blossoms with dehiscing anthers and receptive stigmas. Perhaps more work to define the RNase concentration necessary to completely inhibit pollen development in vitro is needed. Even then, multiple applications may be anticipated to accommodate the duration of bloom and the limited portion of the blossom population which maybe RNase-sensitive at a given time.

DESCRIPTION OF COOPERATION.

Materials and information were exchanged between the groups on a regular basis. Two visits to the US by O.S. facilitated these relationships.

A. niger RNase was produced in Israel and was shipped to the US on a regular basis for laboratory and field experiments.

Oligonucleotides were sent from Israel to the US for the cloning of the

apple S-RNase cDNAs.

The bovine S-Protein cDNA was cloned in Israel and was sent to the US for the construction of the plant transformation vectors. The vectors/transgenic plants will be studied both by the US and the Israeli groups.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

We have determined the optimal fermentation conditions for cost effective production of the *A. niger* RNase from and achieved a cost effective production scale of 20,000 liters. The *A. niger* B1 RNase was isolated to homogeneity and its kinetic and biochemical properties including it's N-terminal sequence were fully characterized. We have gained significant data on the mode of action of the RNase. The field test results both in Israel and California have shown variability in effectiveness and more work is needed to define the RNase concentration necessary to completely inhibit pollen development. Plant transformation vectors expressing anti-sense apple S-RNase genes were constructed with an attempt to produce self compatible transgenic apple trees. Bovine S-Protein cDNA was cloned and successfully expressed in *E.coli*. Plant transformation vector expressing the S-Protein gene was constructed with an attempt to produce transgenic plants expressing S-protein in their style. Exogenous application of S-peptide to these plants will result in active RNase and consequently prevention of fertilization.

CONCLUSIONS

We have been able to develop a cost effective process for the production of *A. niger* B1 RNase and show its effectiveness in inhibiting peach and apple pollen germination, and tube elongation in-vitro, as well as thin fruit in peach and apple, and reduce the number of seeds in citrus. A US patent was recently granted to the Hebrew University for the application of this technology. The fate of this specific application will be dictated by our success or failure better define the RNase concentration necessary to completely inhibit pollen development in field conditions. The plant transformation vectors expressing anti-sense apple S-RNase and the bovine S-Protein gene have great potential for the future development of transgenic plants that will have a built-in genetic control system of their fertilization process. Potential products are transgenic self-compatible apple trees that require no cross-pollination, variety seedless fruits and selective control of fruit set in fruit trees.

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1. Shoseyov O., L. Roiz, U. Ozeri, B. Bravdo and R. Goren. (1996). Methods for the biological control of pollen in plants. U.S. Patent 5,552,139.

APPENDIX

Fig. 1: Map of pBS-Sprot obtained from the Soshvyov laboratory.

Fig. 3: Map of the plasmid pDU96.2601 containing the expression cassette for the Ubi3 promoter.

Fig. 4: Map of pTASprot containing the cloned PCR product of the Sprot gene.

Fig. 5: Map of pDU96.2914 containing the chimeric Sprot gene.

Fig.6: Map of the binary vector pDU95.1001.

Fig. 7: Map of pDU96.3326, the binary vector for the expression of Sprout gene.

Fig. 8: DNA sequence of the Fuji apple S-allele A1.

TCCTGTGCGTCTTCCCCAATACAGAACCAGAAGCATTACTTTGATACAGTAATCAAAATG

S C A S S P I Q N Q K H Y F D T V I K M

430 440 450 460 470 480
TACACAACCCAGAAACAAAACATCTCTGAAATCCTCTCAAAGGCGAATATAAAACCGGGT

Y T T Q K Q N I S E I L S K A N I K P G

490 500 510 520 530 540
AGGAAAAACAGGACACTGGTGGATATTGAAAATGCCATACGTAATGTTATCAACAATATG

R K N R T L V D I E N A I R N V I N N M

550 560 570 580 590 600
ACACCACAATTCAAGTGCCAAAAGAATACTAGGACATCATTGACTGAATTGGTTGAGGTC

T P Q F K C Q K N T R T S L T E L V E V

610 620 630 640 650 660
GGTCTTTGCAGCGATAGCAACTTAACGCAGTTCATAAATTGCCCCCGCCCATTTCCACGA

G L C S D S N L T Q F I N C P R P F P R

670 680 690 700 710 720
GGATCACGGTATTTCTGCCCCACCAATATTCAAGATCGCGGCTAGCTAGTGTGT

G S R Y F C P T N I Q Y - ~~D R G L V G~~

730 740 750 760 770 780
GTGTGTCTGTATGTACACATATAAAATAATAGTAGTTGCCCTATACACGGATGACGATTT

~~V C L V V H I N N S S G P I W C R F~~

790 800 810 820 830 840
CCATATATAGGATGAGAATGTGATTGTAATTCTAACGAACCTGGCTTAATTAATTAATAAT

~~F Y I G E O D S N S N E L G L I N N~~

850 860 870
TAGTATGAATAAAAAAAAAAAAAAAAAA

~~Y E K K K K K K~~

Fig. 9: DNA sequence of the S2 allele of the apple cultivar Royal Gala.

0W8134

CGTATTCAATCGAAAAAATCATTTAGGCTTCTGGAATAGAGAGTGGAACAAACACGGCGC

V F N R K N H L G F W N R E W N K H G A

430 440 450 460 470 480
CTGTGGGTATCCCACAATACGGAACGACTTGCATTACTTTCAAACAGTAATCAAAATGTA

C G Y P T I R N D L H Y F Q T V I K M Y

490 500 510 520 530 540
CATAACCCAGAAACAAAACGTCTCTGATATCCTCTCAAAGGCCAAGATTGAACCGGATGG

I T Q K Q N V S D I L S K A K I E P D G

550 560 570 580 590 600
AAATATCAGGACACAGAAGGAAATTGTAGATGCCATAAGAAAAGGTATCCATGGTAAGGA

N I R T Q K E I V D A I R K G I H G K E

610 620 630 640 650 660
ACCAAACCTCAAGTGCCAAAAGAATACTCAGATGACTGAATTGGTTGAGGTCCTCTTTG

P N L K C Q K N T Q M T E L V E V T L C

670 680 690 700 710 720
CAGCGATGGCAACTTAAAGCAGTTCATAGATTGCCCCCACCATTTTCCAAATGGATCACG

S D G N L K Q F I D C P H H F P N G S R

730 740 750 760 770 780
ACATAACTGCCCCACCAATCATATTCTGTATTAAGAGCCCGCGGCTAGATAGTATATATA
GTGGTTWKTATAAGWCATAATTCT-EcoRI/BamHI

H N C P T N H I L Y - ~~E D A A R V I V~~

0w8 135

790 800 810 820 830 840
CGGGTGACAATTTTCGATATATAGGACGAGAGGAATGAAGTTATGTGGTTGTATTTCTAAT

~~G Q F R Y I S R E E S Y V V V F L M~~

850 860 870 880
GAATAAGTTCTCTTAATTAATTAAACTTATGTGAAAAAAAAAAAA

~~N K F S L I K T V V K K K K~~

Fig. 11: Map of pCRA1 containing the cloned PCR product of the S-allele A1.

Fig. 12: Map of pCRS2 containing the cloned PCR product of the S-allele S2.

Fig. 13: Maps of pHK7 and pCHiP the expression cassette for the ChiP promoter.

Fig. 14: Maps of pDU96.1501 and pUB10P the expression cassette for the *UBQ10* promoter.

Fig. 15: Maps of pCHA1.28 and pCHA1.26 that contain the chimeric A1 in sense and antisense orientations with respect to the ChiP promoter.

Fig. 16: Maps of pUBA1.28 and pUBA1.16 that contain the chimeric A1 in sense and antisense orientations with respect to the UBQ10 promoter.

Fig. 17: Map of pCHS2.11 containing a chimeric S2 in a sense orientation with respect to the ChiP promoter.

Fig. 18: Map of pUBS2.42 containing a chimeric S2 in a sense orientation with respect to the *UBQ10* promoter.

Fig. 19: Map of the binary vector p01UBA1 for expression of the sense A1 regulated by the UBQ10 promoter.

Fig. 20: Map of the binary vector p01UB1A for expression of antisense A1 regulated by the *UBQ10* promoter.

Fig. 21: Map of the binary vector p01UBS2 for expression of the sense S2 regulated by the UBQ10 promoter.