BARD

FINAL REPORT

PROJECT NO. IS-2550-95

**Molecular Mechanisms of Pollen-Pistil
Interactions in Interspecific Crossing
Barriers in the Tomato Family**

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- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Cooperation was complementary since different approaches were used in the two labs to address the common questions.

Abstract

During the evolutionary process of speciation in plants, naturally occurring barriers to reproduction have developed that affect the transfer of genes within and between related species. These barriers can occur at several different levels beginning with pollination-barriers and ending with hybrid-breakdown. The interaction between pollen and pistils presents one of the major barriers to intra- and inter-specific crosses and is the focus of this research project.

Our long-term goal in this research proposal was defined to resolve questions on recognition and communication during pollen-pistil interactions in the extended tomato family. In this context, this work was initiated and planned to study the potential involvement of tomato pollen-specific receptor-like kinases (RLK's) in the interaction between pollen and pistils. By special permission from BARD the objectives of this research were extended to include studies on pollen-pistil interactions and pollination barriers in horticultural crops with an emphasis on citrus. Functional characterization of 2 pollen-specific RLK's from tomato was carried out. The data shows that both encode functional kinases that were active as recombinant proteins. One of the kinases was shown to accumulate mainly after pollen germination and to be phosphorylated in-vitro in pollen membranes as well as in-vivo. The presence of style extract resulted in dephosphorylation of the RLK, although no species specificity was observed. This data implies a role for at least one RLK in pollination events following pollen germination. However, a transgenic plant analysis of the RLK's comprising overexpression, dominant-negative and anti-sense constructs failed to provide answers on their role in pollination. While genetic effects on some of the plants were observed in both the Israeli and American labs, no clear functional answers were obtained. An alternative approach to addressing function was pursued by screening for an artificial ligand for the receptor domain using a peptide phage display library. An enriched peptide sequence was obtained and will be used to design a peptide-ligand to be tested for its effect on pollen germination and tube growth.

Self-incompatibility (SI) in citrus was studied on 3 varieties of pummelo. SI was observed using fluorescence microscopy in each of the 3 varieties and compatibility relations between varieties was determined. An initial screen for an S-RNase SI mechanism yielded only a cDNA homologous to the group of S-like RNases, suggesting that SI results from an as yet unknown mechanism. 2D gel electrophoresis was applied to compare pollen and style profiles of different compatibility groups. A "polymorphic" protein band from style extracts was observed, isolated and micro-sequenced. Degenerate primers designed based on the peptide sequence data will be used to isolate the relevant genes in order to study their potential involvement in SI.

A study on SI in the apple cultivar Top red was initiated. SI was found, as previously shown, to be complete thus requiring a compatible pollinator variety. A new S-RNase allele was discovered from Top red styles and was found to be highly homologous to pear S-RNases, suggesting that evolution of these genes pre-dated speciation into apples and pears but not to other Rosaceae species. The new allele provides molecular-genetic tools to determine potential pollinators for the variety Top red as well as a tool to break-down SI in this important variety.

Molecular mechanisms of pollen-pistil interactions in interspecific crossing barriers in the tomato family.

BARD project No. IS-2550-95

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Final Report

Background, scientific and agricultural relevance of the project

General Background

During the evolutionary process of speciation in plants, naturally occurring barriers to reproduction have developed that affect the transfer of genes within and between related species. These barriers can occur at several different levels beginning with pollination-barriers and ending with hybrid-breakdown. The interaction between pollen and pistils presents one of the major barriers to intra- and inter-specific crosses. The most extensively studied of these are the barriers to intraspecific crosses widely referred to as self-incompatibility (SI). Research on SI in various species has yielded increasing information on genes/enzymes involved in the process however, the complete mechanism has not been elucidated for any one of the SI systems known to date. It is clear though that SI had an enormous adaptive advantage, at least at some stages of evolution, since it appears to have developed independently several times throughout the course of evolution of flowering plants. Compatibility barriers between related species at the pollen-pistil interaction level, or interspecific incompatibility, may have some parameters linked to SI (Murfett et al., 1996) but most likely result from more than one genetic factor affecting proper communication between pollen and pistils as a result of species drifting apart genetically.

Our long-term goal in this research proposal was defined to resolve questions on recognition and communication during pollen-pistil interactions in the extended tomato family. In this context, this work was initiated and planned to study the potential involvement of tomato pollen-specific receptor-like kinases (LePRK) in the interaction between pollen and pistils. By special permission from BARD the objectives of this research were extended to include studies on pollen-pistil interactions and pollination barriers in horticultural crops with an emphasis on citrus.

Scientific and agricultural relevance of the project

The study of inter and intra-specific compatibility presents an interesting and challenging field for basic science research as well as provides knowledge and tools for applied agricultural biotechnology. Establishing the molecular/biochemical basis for interspecific incompatibility may enable overcoming pollination barriers between related species resulting in easier gene transfer between wild species and their related cultivated species. Since much of the breeding for resistance traits relies on introgression of genes from wild species, facilitating this gene transfer would be of significant benefit to agriculture. The mechanisms of intraspecific incompatibility (SI) are of equal interest to science and importance to agriculture. A deeper understanding of the mechanisms involved may allow breakdown of SI in non-parthenocarpic species/varieties to generate plants that produce good yields of fruit without the need to plant rows of pollinators and the reliance on pollinating insects. Also, plants displaying parthenocarpic potential may be manipulated to create incompatibility and to generate seedless fruit.

Results and Discussion

A. Pollen-pistil interactions in the tomato family

We initiated this study after identifying 2 pollen-specific genes encoding putative receptor-kinases. Although we had no functional data, the mature-pollen specific RNA expression pattern and the putative nature of these proteins as transmembrane signaling components suggested a possible involvement in the communication process occurring during pollen-pistil interactions. This study was designed to address the function of these proteins as kinases and to explore a possible role for them during pollen-pistil interaction.

LePRK1 and LePRK2 encode active kinases

Initial determination of kinase activity was achieved by analyzing recombinant enzyme activity. Since trans-membrane proteins are problematic to express in *E.coli*, we opted to work on the intracellular domains of both RLK's. The putative intracellular domains of LePRK1 and 2 were expressed in *E.coli* as MBP (maltose binding protein) fusion proteins (in pMALc2x New-England Biolabs) and as histidine

tagged proteins (pRSETB; Invitrogen). Both the MBP fusion and histidine-tag allowed a single step affinity purification of good yields of soluble protein however, only MBP fusion proteins were active. Both LePRK1 and LePRK2 intracellular domains displayed kinase activity while a mutant version of each (mutated by in-vitro mutagenesis from lysine to arginine in subdomain II of the kinase catalytic region) was not functional (see appendix 1, Figure 8). Based on these results we concluded that both LePRK1 and LePRK2 encode functional receptor-like kinases (RLK's).

Since we were able to obtain soluble fusion protein for both types of fusions (MBP and histidine-tag) it is puzzling that only MBP fusions were active in the kinase assay. Several possibilities could explain this result: (1) The MBP fusion facilitates correct folding of fusion proteins (MBP has been shown to be a superior fusion-protein partner for obtaining soluble and functional recombinant protein – Kapust and Waugh, 1999). (2) The imidazole elutor for the histidine-tag fusion protein elution may be inhibitory for enzymatic activity.

LePRK2 protein accumulates following protein germination while LePRK1 maintains fixed protein level.

Western blots were utilized to monitor the relative level of LePRK1 and LePRK2 protein in mature pollen and in germinating pollen (in-vitro germination). LePRK1 was found to maintain a constant steady-state level of protein, while LePRK2 levels increased dramatically following in-vitro pollen germination (appendix 1 Figure 6). We had previously shown that both RLK's were expressed at the transcript level only late in the development of the pollen grain. The results at the protein level, especially for LePRK2, further support a role for these RLK's after pollen germination.

LePRK2 undergoes phosphorylation in pollen membranes.

Crude membrane fractions were prepared from tomato pollen extracts and assayed for phosphorylation activity. The resulting phosphoproteins were analysed by SDS-PAGE and subjected to immunoprecipitation using LePRK1 or LePRK2 specific antibodies. The results show that only LePRK2 is phosphorylated under the conditions we used (appendix 1, Figure 9A). In-vivo phosphorylation, by incubating mature pollen in labeled phosphate followed by cell lysis and immunoprecipitation seem to show similar results (not shown as the results are not clean enough). We concluded that LePRK2 undergoes phosphorylation in pollen although we cannot

determine whether we observe autophosphorylation or phosphorylation by an unknown kinase. Also, it's not clear (and difficult to test) whether LePRK2 protein is phosphorylated as a result of pollen hydration or some prior developmental signal. An intriguing result was obtained when tomato style extract was added to the in-vitro reaction causing specific dephosphorylation of LePRK2 while other phosphoproteins remained unchanged (appendix 1 Figure 9B and 9D). Several experiments were designed to test the specificity and significance of this dephosphorylation: (1) Tomato leaf protein extracts did not cause the dephosphorylation effect, suggesting that the dephosphorylation response was specific to a component in style extracts (appendix 1 Figure 9C). (2) Dephosphorylation was observed with both *L. esculentum* and *L. pennellii* style extracts showing that there was no species specificity involved. (3) One possibility for an artifact would be the function of some phosphatase from the style extract directly on the intracellular domain of the membrane bound LePRK2. However, this possibility was essentially ruled out since the dephosphorylation result was identical when phosphatase inhibitors (okadaic acid, microcystin) were used or when the style extract was boiled prior to incubation with the pollen membranes. The result obtained using boiled style extracts also suggests that the factor inducing dephosphorylation was probably not a protein.

While the dephosphorylation in the presence of style extract is an interesting result, many questions remain open and unanswered re- its significance. The results suggest some chemical signal transmitted from style extracts to pollen resulting in dephosphorylation of LePRK2; The signal is however not species specific.

Also, most known signals mediated via-RLK's activate kinase activity whereas here we observe dephosphorylation. One possibility is that a specific pollen phosphatase is activated by a component in style extract to dephosphorylate LePRK2. Alternatively, it is possible that the signal from style extract causes shut-down of kinase activity as has been seen in some previous cases (Schweitzer et al., 1995) thereby affecting downstream processes.

Expression of LePRK1 and LePRK2 and phosphorylation studies in yeast.

We have been successful in expressing both LePRK1 and 2 as well as site-specific mutant versions in yeast membranes (appendix 3 Figure 1) using GalI promoter based inducible expression vectors (Foreman and Davis 1994). Each kinase was expressed using a different selectable marker so that co-expression of both kinases was made

possible. Kinase activity was tested both in-vitro (yeast membranes) and by labeling in vivo with phosphate in the presence or absence of style extracts followed by immunoprecipitation. Unfortunately, we were not able to detect phosphorylation activity in-vitro or in-vivo with any of the constructs. To test the possibility that both types of kinases need to be present to obtain activity, double transformants of yeast (containing both LePRK1 and LePRK2) were obtained using the relevant selectable markers. Again, no activity was obtained in-vitro or in-vivo and when challenged with style extract. Since autophosphorylation activity is often found in receptor kinases and since activity was observed in the intracellular portions expressed in E.coli, we concluded that expression of the RLK's in yeast caused them to be non-functional, perhaps due to yeast post-translational modifications. Glycosylation is known to be more extensive in yeast than in plants and may therefore have an effect on the recombinant plant RLK function.

Generation and analysis of transgenic plants.

To establish a functional assay for the pollen specific RLK's, we generated three sets of constructs for introduction into transgenic plants (see appendix 3, Figure 2):

- a) Antisense- constructs contain an inverted cDNA of LePRK1 or LePRK2 under the control of the strong pollen specific LAT52 promoter.
- b) Dominant negative- constructs contain a mutated cDNA (lysine to arginine change in subdomain II of the kinase catalytic region) of LePRK1 or LePRK2 fused to an epitope tag and under the control of the strong pollen specific LAT52 promoter.
- c) Overexpression- constructs contain an intact cDNA of LePRK1 or LePRK2 fused to an epitope tag and under the control of the strong pollen specific LAT52 promoter.

All three constructs were introduced separately into a binary vector cassette containing the β -glucoronidase gene under the control of the LAT59 (moderate) pollen specific promoter. GUS activity may therefore be utilized to detect transgenic pollen and monitor the position effect by the resulting expression level.

The experimental approach the results and the interpretation differed between the American and Israeli labs and are therefore described separately; The American group used a genetic transmission approach to test transgene heritability in

heterozygous plants. The Israeli group opted to screen first for transgene homozygous plants, and to use them to test for pollen function.

Analysis of transgenic plants (American lab)

A total of 190 transgenic plants, for the 6 different transgenic constructs, were analyzed. Surprisingly, almost all of the primary transgenic plants showed an in vitro pollen germination phenotype: the GUS⁺ pollen grains showed reduced pollen tube lengths or no germination, relative to their sibling non-transformed pollen grains (GUS⁻) from the same plant. Genetic analysis (both self-pollinations and outcrosses) showed that most of these plants (see Table 1) had aberrant transmission (i.e. a 2:1 or 1:1 ratio of the linked Kanamycin-resistance Vs. Kanamycin sensitivity) from self pollination, and a <1:3 (sometimes as low as 0:100) ratio from outcrosses to untransformed control tomato. These results suggest that the pollen carrying the transgene cannot transmit the linked Kanamycin resistance gene through the male, and supports the notion that the RLK's have a function during pollen germination. Nonetheless, some plants with no apparent genetic transmission defect (i.e., 3:1 ratio in self-pollination) still showed the in vitro pollen germination phenotype and therefore question our hypothesis. In order to test the correlation between the genetic transmission results and the actual expression levels of LePRK1 and 2 protein, we analyzed transgenic pollen protein by western blot. We chose plants whose progeny gave 3:1, 2:1 and 1:1 genetic ratios. Western blots show no particular correlation between the expression level of LePRK1 or 2 protein and the genetic-transmission ratios. For example, in analysis of the antisense transgenics, some plants with a 1:1 genetic ratio apparently had more LePRK protein than did plants with a 3:1 genetic ratio.

<u>KanR inheritance:</u>	<u>3:1</u>	<u>2:1</u>	<u>1:1</u>
<u>Transgene</u>			
LePRK1	12	3	16
LePRK1M	6	12	25
LePRK1AS	11	4	6
LePRK2	8	7	15

LePRK2M	9	6	17
LePRK2AS	7	15	11

Table 1: Analysis of Kanamycin-resistance inheritance. Numbers in each column indicate the number of independent transformants whose self-progeny gave the indicated genetic ratio.

Analysis of transgenic plants (Israeli lab)

The Israeli lab analyzed transgenic plants of LePRK2 of three different constructs (S-sense overexpression, AS-antisense and M-dominant-negative mutation). We did not detect any unique phenotype using in-vitro germination of transgenic pollen grains. We tried but were not able to distinguish between GUS positive and GUS negative pollen tubes within the style (following pollination) and therefore opted to select transgene-homozygous plants using the linked GUS as a marker for Zygosity and as a marker for position effect. Also an additional generation allowed us to clean out fertility mutations, in some of the plants, that were unrelated to the transgene (presumably somaclonal mutations from the transformation/tissue culture process). In LePRK2-AS (antisense) and LePRK2-M (dominant negative mutation) transgenics, GUS expression was varied with some plants displaying very high expression (50% of the plants for AS and 30% for M); Homozygous plants were obtained from these transgenics showing that transmission of the transgene through the pollen was occurring. However, In LePRK2-S (sense-overexpression) transgenics, GUS expression was always low or not detectable, suggesting that overexpressors with a strong position effect were most probably detrimental. Therefore, no pollen-GUS activity was detected in these plants and no transmission of the transgene was occurring through pollen. We suggest that strong overexpression of LePRK2 caused pollen abortion due to transmission of an unnatural signaling cascade in the immature pollen grain. Overexpression of receptor-kinases is known from mammalian systems to cause transmission of signaling cascades in the absence of a ligand.

Pollen from homozygous plants of the three classes (LePRK2 “over-expresser” homozygous are weak expressers) was analyzed for in-vitro germination and in-vivo pollination compared to wild-type pollen. General compatibility of the pollen to *L. esculentum* and *L. pennellii* styles was tested, as well as the speed of growth through the style. Transgenic pollen of homozygous plants was just as efficient as wild type

pollen in the in-vitro germination and in growth through *L. esculentum* styles. Also, transgenic pollen, like wild-type pollen, was not able to overcome incongruity in *L. pennellii* styles.

Based on these results we suggest the following interpretations and possibilities:

- 1) Overexpression (medium-high levels) of LePRK2 during pollen development is detrimental for the pollen grain, probably by activating a signaling cascade at the wrong time. It therefore may have been beneficial to use a weak-moderate promoter (rather than the very strong LAT52 promoter) to drive the sense-overexpression constructs.
- 2) Dominant negative mutation transgenic pollen (LePRK2-M) did not affect pollen function. By design, these mutations are effective only if the RLK functions as a dimer/multimer allowing the mutant protein to dimerize with an endogenous RLK. Since we have no data on the mode of action of LePRK1 and LePRK2 (ie. Monomer, dimer), it is possible that they function as monomers. In this case dominant-negative mutation transgenes would not be expected to produce a phenotype.
- 3) Antisense LePRK2 transgenic pollen did not affect pollen function. Since RLK's are at the top of signaling cascades it is possible that they don't need to be present in optimal levels and that lower levels are sufficient for normal function. Indeed in western blots conducted on pollen protein from these plants we always saw some LePRK2 protein present. Perhaps we did not screen enough transgenic AS plants to identify a plant with sufficient LePRK2 protein reduction. Alternatively, other RLK's may have an overlapping function that can compensate for lack of normal levels of LePRK2 protein (ie. redundancy).

Screen for putative ligand

In the lack of clear phenotype in the transgenic plants, identification of a ligand may be the key to studying function of the RLK's LePRK1 and LePRK2. While isolation of a real ligand is obviously preferred, the lack of knowledge on the nature of the ligand (ie. proteinaceous or other) directed much of our efforts on finding an artificial peptide ligand via screening a phage-display library. The theoretical advantage of peptide ligands is that they may mimic the action of virtually any kind of ligand (ie. protein or other). To facilitate the bio-panning screen for a ligand, the extracellular (putative ligand binding) domain of LePRK2 was expressed in *E. coli* in frame with a

carboxy terminal histidine tag which allowed affinity purification of the protein. The recombinant extracellular domain protein was used to coat a polystyrene petri-dish. The dish, in turn, was used for 3 rounds of the biopanning process using a random 7mer combinatorial peptide library. Five of the resulting clones were sequenced and the results are summarized in table 2. Apart from clone #4, there appears to be a limited consensus pattern: Y (or F), Hydrophobic, T (or S), X, X, D (or E). We concluded that either more cycles of biopanning are necessary or that the specificity that can be generated from a short peptide sequence cannot result in a more defined consensus sequence. A new experiment with more biopanning cycles will determine whether a more defined consensus can be reached. Based on these results an artificial peptide will be synthesized to study its effect on pollen germination in-vitro.

Clone #	Peptide sequence
1	H Y V T V M D
2	F V S T I D G
3	Y L T L N E A
4	M I N T L Q N
5	G Y I T A I D

Table 2: Peptide sequences resulting from phage-display screen on LePRK2 extracellular domain. Conserved amino acids are presented in bold.

B. Studies on pollen-pistil interactions in citrus

Self-incompatibility (SI) has been noted for several species/varieties of the citrus family. Its importance in citrus is 2-fold; (1) As an important factor in yield in some non-parthenocarpic varieties that may be used for juice (where seed abundance is not a serious drawback). (2) As a natural mechanism that may be manipulated in the future to induce seedlessness in parthenocarpic varieties intended for fresh-fruit consumption.

Establishment of compatibility relations within the species *citrus grandis* (pummelo)

We chose to study citrus-SI in Pummelo since it is considered as one of the three “founding” species of the citrus family and since there were documented reports of seedless fruits obtained in some varieties grown in solid blocks. Pummelo was domesticated in several areas in east-asia and several of the varieties are traceable to distinct groups representing common geographic origin. We chose to study compatibility relationships between (and within) 3 varieties of Pummelo of those that were available to us; Var. Chandler (Thai group) Var. Yehiam (Chinese group) Var. Tahiti (not classified into a clear group). The physiological study involved the following steps:

- A) Seed-count in fruits of self and cross-pollinated varieties.
- B) Analysis of pollen tube growth in-vivo in the style of self and cross-pollinated varieties.
- C) Analysis of pollen tube growth and the potential of displaying SI in-vitro.

Pollination compatibility relations among and within 3 pummelo varieties were initially established by self and cross pollinations and seed count of the developing fruit. Pre-anthesis flowers were emasculated, pollinated with the relevant pollen and bagged to prevent contaminating pollen. The results (representing 2 years of crosses) summarized in Table 3 show that each of the 3 varieties is self incompatible but cross-compatible with the other 2 varieties. Its important to note that self pollinations often resulted in flower abscission and there seemed to be different parthenocarpic potential for the three varieties.

Pollen: Style	Chandler	Tahiti	Yehiam
Chandler	1.8 ± 1.2	29.8 ± 9.1	28.3 ± 12.7
Tahiti	25.8 ± 5.1	2.9 ± 1.5	26.7 ± 4.5
Yehiam	23.2 ± 3.3	22.6 ± 2.6	1.5 ± 1.1

Table 3: Pollination compatibility analysis by fruit seed count. Average seed count is presented for each cross.

Pollen-tube growth in the style was monitored by fluorescence microscopy of aniline-blue stained sections of pollinated styles. We experimentally determined that it takes 6-7 days for the pummelo pollen tube to grow throughout the style and reach the ovules in a compatible pollination. Therefore all subsequent self and cross pollinated styles were harvested after 7 days followed by treatment for fluorescence microscopy. Each of the 3 varieties was self and cross pollinated as described above; The results presented in appendix 3-figure 3a,b,c,d show the following:

- (1) In all combinations of cross pollination strong germination was observed and directional growth of large amounts of pollen tubes towards the ovary was seen in all parts of the style. The presence of many pollen tubes in the vicinity of the ovules was clearly seen and shows a compatible interaction.
- (2) In all self-pollinations strong germination was also observed however, much fewer pollen tubes were observed in the upper-style growing directionally towards the ovary and these were all arrested by the mid-style. The picture of all self-pollinations shows the presence of self-incompatibility.

Initial working hypothesis for citrus self-incompatibility (SI)

According to the phylogenetic map of Takhtajan (1969,1997), the order *Rutaceae* is rather distant from any species from which molecular evidence on SI is already available. Nevertheless, it appears that the *Rutaceae* is on a side-branch of the main evolutionary branch “connecting” the *Solanaceae* and the *Rosaceae*, both of which are known to comprise stylar T2 type RNase alleles in their incompatibility systems (S-RNases). We therefore tested the possibility that a similar S-RNase-based incompatibility system is present in citrus:

- RNase activity gels were employed to detect T2-type RNase activity in pummelo styles. A Protein band of 25-30KDa displaying RNase activity was detected in styles of all three species of pummelo, suggesting that T2-type RNase was present (not shown).
- Degenerate primer based RT-PCR was employed to isolate style expressed T2-type RNases from styles of the three pummelo varieties. A highly conserved region of all T2-type RNases was used to design a degenerate primer encoding the amino-acid sequence: F T V/I H G L W P. Only one type of cDNA was isolated from Tahiti style mRNA (appendix 3 Figure 5a) and completed by 5'-RACE (by the method described in appendix 2). An almost identical cDNA was present in the other 2 varieties. The deduced amino acid sequence displays high homology to Arabidopsis RNS2 S-like RNases (Taylor et al., 1993), which are similar proteins but not involved in SI (see appendix 3 Figure 5a). A phylogenetic analysis of S-RNases and S-like RNases places the citrus sequence as part of a distinct group comprising the S-like RNases (Figure 5b). Based on the homology to S-like RNases from various distant species as well as the fact that only one allele was found in three varieties of pummelo, it seems likely that the citrus gene we isolated is not involved in self-incompatibility. Also, since we conducted an exhaustive screen, it seems likely that no additional S or S-like RNases are present in pummelo styles.

Non-biased approach to identify components of the SI machinery in citrus

In parallel to the search for S-RNase incompatibility components, we initiated a non-biased approach to detect potential components of the citrus SI system. Protein profiles of style and pollen tissue of the 3 pummelo varieties were compared by 2-D gel electrophoresis (IEF followed by SDS-PAGE) using a non-equilibrium system which allows representation of both acidic and basic proteins. Pollen Protein profiles of the 3 varieties seemed to be identical (data not shown) and no "polymorphic" protein spots were detected. However, the style protein profile displayed a difference between the varieties examined (see general profile of style-protein of the variety Tahiti – appendix 3, Figure 4a and a comparison of profiles of varieties Tahiti and Chandler- appendix 3, Figure 4b). The variety Tahiti displayed 2 spots of similar molecular weight but slightly different PI. In contrast, the variety Chandler displayed only the higher PI spot. Furthermore, the intensity of the Chandler spot (relative to the Tahiti spots) may suggest that it consists of a double dose of the same protein,

meaning that either Chandler is homozygous for this allele or that 2 very similar alleles are co-migrating to a very close position in the 2-D gel (see Figure 4b). The profile obtained with Yehiam style proteins was also “polymorphic” for the same spot/s and showed only the lower PI spot (not shown).

Both protein spots from the variety Tahiti were isolated and subjected to microsequencing (N-terminal and internal) resulting in the following sequences:

(1) Higher PI spot: N-terminal- (M/D/H)VNAIAx(V/I)

Internal sequences: AxGVIGLLSY; AIVIHKD

(2) Lower PI spot: N-terminal- (M/T/G)xVP(P/H)HxAD

Work is under way to isolate cDNA fragments based on these partial-peptide sequences.

C. Self-incompatibility in apple

Gametophytic self incompatibility in the Rosaceae is well known and established and compatibility groups have been determined for many of the fruit-trees of this group including apples. In more recent years part of the molecular mechanism governing SI in the Rosaceae has been discovered and shown to involve alleles of RNases (S-RNases) specifically expressed in the styles (Broothaerts et al., 1995); the pollen component of SI remains unknown. The family of known alleles in apples and pears, consists of more than 20 known to date. These known alleles contribute molecular tools to easily determine genetically compatible pollination partners. However, some varieties, including commercially important ones, contain unknown alleles. Identification and isolation of these alleles would contribute significantly to determination of the correct pollination partners.

Isolation of a new self-incompatibility allele from Top-red apples

The variety Top-red is an important commercial variety in Israel which displays complete self-incompatibility. Therefore, to obtain fruit, a proper pollinator row needs to be planted every few rows and special care must be taken to ensure the presence of pollinators during the flowering season. However, based on a fingerprinting-type analysis only one of its alleles is a previously known allele (Goldway et al., 1999). We employed degenerate primer based RT-PCR on style mRNA to isolate and

sequence the putative unknown allele. Sequences conserved between *Malus* and *Pyrus* S-RNase alleles were used to design the degenerate primer which encodes the amino-acid sequence: Q F T Q Q Y Q. The resulting cDNA sequence we isolated (appendix 3 Figure 6a) encodes a previously unknown allele highly homologous to the Japanese pear S4 allele (87% identity) and S1 allele (84% identity). Since the most homologous apple allele (S26) is only 66% homologous, we conclude that divergence of incompatibility alleles occurred before speciation to apples and pears. Indeed, a phylogenetic analysis of S-RNases of apples and pears gives a mixed picture of alleles not classified by species (appendix 3, Figure 6b). Interestingly, alleles of other members of the Rosaceae: almond and cherry, seem to comprise a distinct group of their own, not more related to alleles of apples and pears than to alleles of Solanaceous species (Figure 5b). This point raises interesting questions on the evolution of S-RNase incompatibility alleles, especially since it has previously been suggested that S-RNase based incompatibility developed separately in the Rosaceae (Sassa et al., 1996). The high similarity of the new Top red allele to pear S4 and S1 alleles is noteworthy but not unique since even higher identity was found for alleles S11 and S13 of *Solanum chacoense* (Matton et al., 1999). Both cases show that development of new incompatibility alleles is an ongoing evolutionary process and that relatively few amino-acid changes can determine unique compatibility groups. Isolation of the new allele provides the molecular-genetic tools to determine proper pollinators for the variety Top-red. Also, it may facilitate future work to break-down SI in this variety using a molecular approach.

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Publications

- Neumann H., Frydman A. and Eyal Y. Self-incompatibility and cross-compatibility relations in *Citrus grandis* (pummelo).
In preperation. (relevant figures in appendix 3)
- Neumann H., Flaishman M., Frydman A. and Eyal Y. Identification of a new allele of self-incompatibility from the apple cultivar Top red; Evolutionary implications.
In preperation. (relevant figure in appendix 3)
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Biotechniques 27: 656-658. See appendix 2
- Muschietti J., Eyal Y. and McCormick S. (1998) Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK1 and LePRK2.
The Plant Cell 10: 319-330. See appendix 1