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PRELIMINARY INVESTIGATION OF THE MOLECULAR COMPONENTS RESPONSIBLE FOR THE SELF-INCOMPATIBILITY RESPONSE OF SWEET CHERRY

presented by

Thomas S. Brettin

has been accepted towards fulfillment
of the requirements for

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Major professor

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PRELIMINARY INVESTIGATION OF THE MOLECULAR COMPONENTS RESPONSIBLE FOR THE SELF-INCOMPATIBILITY RESPONSE OF SWEET CHERRY

By

Thomas S. Brettin

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Submitted to
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ABSTRACT

PRELIMINARY INVESTIGATION OF THE MOLECULAR COMPONENTS RESPONSIBLE FOR THE SELF-INCOMPATIBILITY RESPONSE OF SWEET CHERRY

By

Thomas S. Brettin

Self incompatibility (SI) in flowering plants prevents self pollination thereby promoting outcrossing. While this may be advantageous for the survival of the plant species, it presents a problem to farmers and breeders. Crops which are dependent on fruit resulting from successful pollination must rely on cross pollination.

In order to better understand the molecular components of the self incompatibility response in sweet cherry (*Prunus avium* L.), stylar proteins were investigated using different electrophoresis protocols. Nucleic acid analysis using PCR and degenerate primers was employed to isolate DNA sequence which might code for an allele of the SI locus, which have been reported to be ribonucleases in both *Rosaceae* and *Solanaceae* families.

The work presented here describes the characterization of stylar proteins and the isolation of cDNA which codes for a sweet cherry ribonuclease.

To my wife Sherry and my daughter Sarah.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank all those who made this thesis possible. In particular, Dr. Amy Iezzoni for allowing me to work in an independent environment while providing the necessary support when it was needed. I would also like to thank Wendy Huss for her seemingly endless technical support of the research presented herein.

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INTRODUCTION

Gametophytic self incompatibility (SI) is the most prevalent mechanism preventing self pollination in flowering plants. Gametophytic SI is commonly controlled by a single nuclear gene (*S*-locus) with multiple alleles. Self fertilization is prevented by inhibition of pollen tubes that have the same incompatibility genotype (*S*-allele) as that of the pistil on which they have germinated.

Recently, molecular investigations have increased our understanding of the stylar component of the *S*-alleles in plants from the *Solanaceae* family. Using cDNA clones of the stylar *S*-alleles from tobacco, it has been shown that the *S*-allele products are glycoproteins that are secreted by cells of the stylar transmitting tissue (Cornish et al., 1987). The *S*-allele glycoproteins have been identified as ribonucleases which degrade the pollen tube RNA of pollen with like *S*-alleles (Gray et al. ,1991). Amino acid sequence comparison between 6,3, and 1 *S*-allele(s) from *Nicotiana alata*, *Petunia inflata*, and *Solanum tuberosum*, reveal a highly conserved region at the amino terminus and

three hypervariable regions (Haring et al., 1990). The pollen product of the *S*-locus is still not known.

Sweet cherry (*Prunus avium* L.) is one of the more important agricultural crops exhibiting gametophytic SI with six *S*-alleles identified in breeding experiments (Way, 1967). Because cross pollination is required for fruit set, at least two cross compatible cultivars must be planted in alternate rows in orchards and bees provided as pollinators. Frequently, the two complementary cultivars have different maturity dates making harvesting and spraying less efficient. Yields are often reduced due to limited egg viability and poor pollination since bees remain in their hive when the wind velocity is greater than 25 kilometers per hour and/or when the temperature drops below 10°C.

Because of the inefficiencies and yield reduction associated with growing SI sweet cherry cultivars, self compatibility is one of the most important objectives in sweet and sour cherry breeding programs. Self compatibility in sweet cherry was obtained by x-ray radiation applied to Napoleon (S_3S_4) flower buds at the pollen mother cell stage and subsequent selection in S_3S_4 styles (Lewis and Crowe, 1953). Breeding experiments confirmed that there was a mutation at the S_4

locus which affects only pollen activity (i.e. pollen with the mutated S_4 alleles is able to fertilize plants with a normal S_4 allele and not vice versa)(Lewis and Crowe, 1956). Currently sweet cherry breeders must wait until the seedlings flower (3-5 years) before determining if individual seedlings are SI by laborious controlled pollination of bagged flower clusters and subsequent analysis of fruit set or pollen tube growth in fixed styles. Because bloom may last for only 5 days, SI evaluations of large numbers of seedlings is impossible. If the *S*-alleles could be identified in hybrid seedlings soon after germination, it would reduce the orchard space required for the seedling populations and permit breeders to evaluate SI from a large number of seedlings.

SI in sweet cherry is similar to that in the *Solanaceae*. The initial experiments that associated glycoproteins with stylar *S*-alleles were done with sweet cherry (Mau et al., 1982; Williams et al., 1982). An antigenic glycoprotein, isolated from Napoleon styles, was able to inhibit in vitro growth of self-pollen. The isolated glycoprotein contained at least two closely related major components which were suggested to correspond to the products of the two *S*-alleles. More recently, northern analysis using mRNA from

styles of sweet cherry probed with cDNA from a *N. alata S*-allele failed to identify a homologous product (A. Clarke, pers. comm.); however, this was not unexpected because of the limited homology between different *S*-alleles within a species (Anderson et al., 1986).

The objective of this study was to identify the glycoproteins and cDNAs corresponding to the S_4 and/or S_3 alleles from the sweet cherry cultivar Emperor Francis. Two approaches were used: (1) identify glycoproteins which segregated with known S-alleles [Chapter 1], and (2) design primers to conserved regions of known *S*-alleles and use a PCR based method to obtain RNases from both genomic DNA and stylar cDNA [Chapter 2].

LITERATURE REVIEW

Gametophytic Self Incompatibility in Sweet Cherry

Commercial cultivars of *Prunus avium* L., sweet cherry, are unable to self pollinate (Crane and Brown, 1937). In addition to self incompatibility, many cultivars are cross incompatible. Commercially important cultivars Napoleon, Bing, Lambert, and Emperor Francis are cross incompatible with each other. Based on these cross incompatible observations, different incompatibility groups were constructed. A comprehensive listing of cultivars and their associated incompatibility group was assembled by Knight (1969). A more recent study by Tehrani and Lay (1991) investigated incompatibility grouping of sweet cherry cultivars from Vineland, Ontario. Lansari and Iezzoni (1990) have extended this analysis to the related sour cherry species *Prunus cerasus*.

The genetic basis of self and cross incompatibilities was described by Crane and Lawrence (1929) and Crane and Brown (1937). Their findings showed that cherry incompatibility is due to a single gene (called *S*-gene) which prevents

normal pollen tube growth,thereby preventing pollination. The incompatibility response was seen when the haploid pollen carries a particular *S*-gene which is present in the somatic tissue of the female pistil.

Self compatibility has been achieved in sweet cherry by applying x-ray radiation to Napoleon flower buds at the pollen mother cell stage and subsequent selection in S_3S_4 styles (Lewis and Crowe 1953). Breeding experiments by Lewis and Crowe (1956) confirmed that there was a mutation in the S_4 allele which affects only pollen activity (i.e. pollen with the mutated S_4 allele is able to escape the incompatibility mechanism when presented on a S_4 allele containing pistil). The resulting self-compatible cultivar, Stella, has not lived up to commercial expectations and hence does not appear in commercial orchards.

In the early 1980's, the investigation of self incompatibility in sweet cherry turned to the molecular level. As a first step, Raff and Clarke (1981) and Raff et al. (1981) demonstrated the presence of components in the S_3S_4 styles which were associated with this *S*-allele group. Both of these components were shown to be unique to the S_3S_4 group immunologically using antiserum raised against style

extracts. Mau et al. (1982) began isolating and characterizing components of sweet cherry styles which might be associated with a self-incompatibility genotype using this antigenic information. Their findings consisted of two antigenic glycoproteins, one which was style specific to all *Prunus* species tested (Antigen P),and one which was associated with the self-incompatibility genotype (Antigen S). The isolated glycoproteins had a substantial carbohydrate content (Antigen P 17.2%; Antigen S 16.3%),and had apparent molecular weights of 32,000 Da (Antigen P) and 37,000-39,000 Da (Antigen S). Antigen S was found to be secreted into the medium of suspension cells raised from both leaf and stem of *Prunus avium*. Two dimensional electrophoresis of I^{125} labeled Antigen S showed two components of similar molecular weight (~37,000 Da) on the alkali side of the gel. The same experiment with Antigen P indicated a single acidic component. It was eluded to that the two components of Antigen P could be alleles of the *S*-locus.

Further studies by Williams et al. (1982) demonstrated the effect of Antigen P and Antigen S on *in vitro* pollen tube growth. Their findings supported the role of Antigen S in the incompatibility response. Antigen S displayed the

ability to inhibit pollen tube growth by 65% at a concentration of 20 ug/ml. None of the other style components tested were effective pollen tube growth inhibitors including Antigen P.

Investigators of this group then turned their attention away from *Prunus* and began to focus on a more favorable biological system, *Nicotiana alata.* This marked the end of active research into the molecular components of sweet cherry self incompatibility until present day.

Gametophytic Self Incompatibility in the *Solanaceae* Family

Investigations into the molecular mechanism of gametophytic SI turned to the *Solanaceae* family. Different species were investigated with the most effort going into *Nicotiana alata* by the lab of Adrienne Clark and *Petunia hybridia* by the labs of Jan Vendrig and Teh-hui Kao. The following review of the *Solanaceae* family will consider the findings of these two species separately, although much information was obtained concurrently for these two species. Finally, I will summarize some of the smaller projects ongoing in different species in the *Solanaceae* family.

News of progress ceased from 1982 (Williams et al.) on the sweet cherry work until a major article emerged in Nature.

Anderson et al. (1986) reported the first successful cDNA cloning of a stylar glycoprotein associated with the self-incompatibility phenotype of *Nicotiana alata*. They set forth as criteria for positive identification of an *S*-allele that, first, its presence in reproductive tissue must be consistently associated with the corresponding breeding behavior of the plant, and second, that it should show specific biological activity under an appropriate bioassay system (for example, it should inhibit pollen tubes bearing the same *S*-allele, but not tubes bearing a different *S*-allele). In their work, they demonstrated genotypic specificity of the *S*-allele protein by two dimensional electrophoresis of 53 plants resulting from reciprocal crosses involving two genotypically different plants. Complete co-segregation of the *S*-allele protein with the observed phenotype was shown. The resulting *S*-allele proteins were characterized as having a molecular weight of about 32,000 Da and an isoelectric point of greater than 9.5.

Subsequent amino acid sequencing of the N-terminus of the isolated protein provided degenerate DNA sequence information, which was used to identify a cDNA clone. Expression analysis of this clone showed both temporal and

spatial expression patterns consistent with the timing and location of the incompatibility response.

Continued pursuit of the identification of more *S*-alleles in *N. alata* lead to the identification of new alleles (Anderson et al. 1989, Jahen et al 1989a, Jahen et al. 1989b). Amino acid sequence comparison of the three existing sequences showed regions of high similarity and regions of high variability. Southern analysis showed co-segregation of the identified cDNAs with the observed phenotype. The approach to the identification of new cDNAs was essentially the same, whereas highly conserved N-terminal amino acid sequence information was used to identify the candidate cDNA clone. Jahen et al. (1989a) employed a new protein purification technique, fast liquid protein chromatography (FPLC), on stylar extracts to prepare protein for N-terminal sequencing. Biological activity was demonstrated by the development and use of an *in vitro* pollen tube growth assay. Although this pollen tube assay had significant improvements compared to the assay used in the early 1980's on sweet cherry pollen, there was still some cross reactivity which was inhibiting heterologous pollen (i.e. S_3 glycoprotein inhibited not only S_3 pollen tubes but also S_2 and S_6 pollen

tubes). Inhibition was measured in the presence of 300 to 600 ug/ml of the appropriate *S*-glycoprotein.

At this time, interest was mounting on the possibility that the N-linked glycans on the *S*-glycoproteins could be the variable component that conferred allele specificity. The methods were in place to isolate *S*-glycoproteins in pure form (Jahen et al. 1989b). Woodward et al. (1989) underwent investigations to characterize the glycan chains of five different *N. alata S*-glycoproteins. In summary, their results were indicative of heterogeneity in the structure of the glycan chains. The authors go on to recognize that if allelic specificity were to reside in the fine structure of the glycan chains attached to the protein, then an additional controlling factor could play a role in determining this fine structure, and for genetic reasons, would have to be tightly linked to the *S*-locus. Alternatively, if the allele specificity of the *S*-glycoprotein resides in the position of the glycan chains relative to the protein, the order and number of glycosolation sites within the protein would be the determining factor.

Progress on the biochemical mechanism of self incompatibility took a large step forward with the discovery of a ribonuclease activity present in the *S*-glycoproteins. Sequence analysis had shown that three alleles of the *S*-gene of *N. alata* (Anderson et al. 1986, Anderson et al. 1989) encode style glycoproteins with regions of defined homology. Two of those homologous regions displayed precise homology with RNase T_2 and RNase Rh. McClure et al. (1989) showed that five known S-alleles (S_1, S_2, S_3, S_6, and S_7) not only contained the region of homology to RNase T_2 and RNase Rh, but also displayed ribonuclease activity when assayed as described by Brown and Ho (1986).

Soon after the reports that isolated *S*-alleles had an associated ribonuclease activity, McClure et al. (1990) hypothesized ribonuclease activity as the biochemical process by which pollen tube growth is arrested. This work was based on *in vivo* observations that P^{32} labeled rRNA isolated from styles was degraded when incompatible pollen was present and the same labeled rRNA was not degraded when compatible pollen was present. These observations lead to the proposal of a model in which the gametophytic self incompatibility system in *N. alata* acts through a cytotoxic mechanism directed against pollen RNA.

The action of the ribonuclease activity of isolated *S*-proteins on pollen tube growth was investigated by Gray et al. (1991). Their results indicated that the selectivity of the degradation was due to selective uptake of *S*-protein by the pollen tube and not due to selective degradation of RNA. *S*-proteins inhibited *in vitro* translation of pollen tube RNA in a non-specific manner in wheat germ cell free extracts. A particularly interesting finding of this study, and perhaps unexpected, was that heat treating S_2-RNase largely destroyed the ribonuclease activity of the protein, but did not reduce the inhibitory effect on *in vitro* pollen tube growth. Heat treated S_2-RNase accumulation on the outer surface of the pollen grains was greatly increased compared to non-heat treated S_2-RNase. It was suggested that this increased accumulation was responsible for *in vitro* pollen tube growth inhibition, and that the *in vitro* and *in vivo* systems needed to be compared with caution. This concern was certainly valid since earlier *in vitro* experiments had shown cross reactivity of some *S*-proteins.

Early attempts to create a transgenic model focused on transforming *Nicotiana tabacum* with both *N. alata* S_2 cDNA and genomic DNA (Murfett et al. 1992). The hope was that *N. tabaccum* would express the transgene in both pollen and

pistil, hence creating an incompatibility mechanism in an otherwise self-compatible plant. Transgene expression was obtained in the pistil before, during, and after anthesis, and transgene expression was detected in anthers prior to anthesis. Expression levels in the transgenic plant were significantly lower than expression levels in transgene donor (approximately 100 fold lower). There was no observed change in the compatibility type of the transgenic *N. tabaccum*. This result was presumably due to the inability to reach wildtype expression of the S_2 allele in *N. tabaccum*.

This summarizes the vast progress made by Adrianne Clark and her associates in understanding the stylar gametophytic SI gene in *N. alata*. Work done by these investigators opened the door to molecular SI investigations in other members of the *Solanacea* family such as *Petunia inflata*, *Lycopersicon peruvanium*, and *Solanum tuberosum*.

In 1990, publications from the lab of Teh-hui Kao reported the isolation of previously unidentified *S*-alleles of *N. alata*. Kheyr-Pour et al. (1990) identified four alleles, S_1, S_a, S_{F11}, and S_z and compared the deduced amino acid sequence of these and three previously identified *S*-

proteins. Their findings revealed 53.8% homology among the seven proteins. There were 60 conserved residues, including 9 cystines, and 144 variable residues. Of the 144 variable residues, 50 were identified as hypervariable based on their Similarity Indices. The conserved and hypervariable residues could be clustered into five regions. It was this observation that led to the hypothesis that, since the hypervariable regions accounted for most of the inter-sequence variability, these hypervariable regions could be responsible for the allelic specificity.

A year earlier, Broothaerts et al. (1989) identified the first *S*-proteins of *P. inflata* (S_1, S_2, and S_3). The proteins were isolated by a combination of ConA-Sepharose and cation exchange FPLC. These proteins were then checked by co-segregation analysis. The N-terminal amino acids were determined and compared to those of *S*-proteins from *N. alata*. Homology in this region led to the hypothesis of a common cellular membrane transport peptide for *N. alata* and *P. inflata*. These new *Petunia S*-proteins differed slightly in molecular weights (S_1 27000, S_2 33000, and S_3 30000 Da) and isoelectric points (8.7, 8.9, and 9.3 respectively). The distribution of these proteins in the pistil showed the proteins were present in high concentration in the style and

the uppermost part of the stigma. Time course analysis showed an increase in the amount of the proteins during flower maturation. These observations were consistent with earlier findings in *N. alata*. Finally, these *S*-proteins were shown to have ribonuclease activity (Broothaerts et al., 1991) and that this ribonuclease activity was still present after deglycosylation of the *S*-protein with the enzyme peptide-N-glucosidase F (PNGaseF). This suggested that the role of the glycan moieties was other than the ribonuclease function.

Identification of the cDNA clones representing the S_1, S_2, and S_3 alleles of *P. inflata* (Ai et al., 1990) was accomplished using the S_2 cDNA clone from *N. alata* as a probe against stylar cDNA libraries of *P. inflata*. This was the first successful cross species hybridization experiment. Spatial and temporal expression was consistent with previous studies.

Ai et al. (1992) approached the question of self incompatibility by investigating a self-compatible cultivar of *P. hybridia*. They cloned two *S*-allele-like cDNAs which displayed all the previously mentioned characteristics of an *S*-allele. Homology in the protein N-terminus with previous

S-protiens was identified. These *S*-proteins had ribonuclease activity, and spatial and temporal expression was consistent. The investigators elude to the disruption of the self recognition mechanism as the cause of the self compatible phenotype.

Genomic sequences of the flanking regions of two *P. inflata* *S*-alleles (Coleman and Kao, 1992) were cloned and shown to contain heterogeneous and repetitive sequences. They reported that the sequence diversity in the flanking region increased to the point that interallelic homology completely disappears. This lack of homology lead Coleman and Kao to conclude that the two alleles have evolved independently as the result of an unknown mechanism which suppresses recombination in this region of the chromosome.

Much of the discussion up to now was about the isolation and characterization of *S*-proteins and their corresponding cDNAs. These experiments were all based on co-segregation analysis of the protein or cDNA with the corresponding phenotype. This type of analysis does not exclude linkage disequilibrium, and thus does not provide definitive proof for the phenotypic effect of these proteins.

In the largest breakthrough since the first cloning of a *S*-allele cDNA, Lee et al. (1994) showed definitively that *S*-proteins control rejection of incompatible pollen in *P. inflata*. Experiments using both loss-of-function and gain-of-function approaches to ascertain whether previously identified *P. inflata S*-proteins were responsible for self incompatibility interactions between the pistil and pollen showed in fact that this was the case. Loss-of-function was observed when S_3 antisense constructs were used to transform plants of the S_2S_3 genotype. Subsequent pollination of the transgenic plants showed that S_3 pollen was no longer rejected. This breakdown in the incompatibility response was concordant with the disappearance of S_3 mRNA and the S_3 glycoprotein.

The gain-of-function experiments were based on the transformation of an S_1S_2 plant with a S_3 construct and subsequent pollination tests to ascertain the viability of S_3 pollen on the transgenic pistils. As expected, the transgenic plant rejected S_3 pollen. When self pollination was attempted at the immature bud stage, these transgenic plants set fruit. Thus, their failure to set fruits when pollinated with S_1, S_2, and S_3 pollen was a true self-

incompatible response, and not due to female sterility resulting from tissue culture manipulation.

In conclusion, the results of the transgenic experiments provided direct evidence *in vivo* that the *S*-proteins of *P. inflata* were necessary and sufficient for the pistil to reject self pollen.

This discussion has focused on the work done in *N. alata* and *P. inflata*. The former provided the scientific community with the first cDNA clone of a *S*-allele, the later provided the definitive proof that these *S*-alleles were responsible for self incompatibility. Work in other species of the *Solanacea* family includes cloning of *S*-alleles in *Solanum chacoense* (Xu et al., 1990), cloning of a *S*-allele in *Lycopersicon esculentum* (Chung et al., 1993), and investigations of a self compatible mutation in *Solanum tuberosum* (Thompson et al., 1991).

Gametophytic Self Incompatibility in Other Plants

Poppy, *Papaver rhoeas*, displays classical genetic gametophytic self incompatibility. Franklin-Tong et al. (1991) examined poppy stylar proteins and were unable to find any evidence for the involvement of a ribonuclease in

the self incompatibility response. Their conclusions were based on two major experiments. The first was a developmental expression analysis of the pistil for ribonuclease activity during the maturation of the pistil. As noted earlier, the level of expression of ribonuclease activity increases with the maturation of the pistil in *N. alata* and *P. inflata*, reaching a maximum when pollination occurs naturally. This was not the case with *P. rhoeas*. Franklin-Tong et al. (1991) found no significant difference in the level of expression during pistil maturation. The second argument against ribonuclease involvement was based on pollen inhibition assays. They tested protein fractions from the pistil known to contain biologically active inhibitors of *in vitro* pollen tube growth for ribonuclease activity. Those fractions which displayed the greatest ability to inhibit pollen tube growth *in vitro* contained no significant amounts of ribonuclease activity. The authors point out that one notable difference between *P. rhoeas* and *N. alata* is the site of pollen inhibition. In *P. rhoeas*, the site of inhibition is on the stigma, while in *N. alata*, the site of inhibition is the style. Hence, while genetically similar to the gametophytic incompatibility system of *N. alata*, this system resembles the sporophytic

incompatibility system of *Brassica oleracea* in that the site of inhibition is on the stigma.

Self-incompatibility related ribonucleases have been identified in the styles of *Pyrus serotina* (Japanese Pear) by Sassa et al. (1992). In their work, isoelectric focusing of style protein extracts on polyacrylamide gels, followed by ribonuclease activity staining of the gel, revealed basic ribonuclease isoforms which correlated with the known *S*-genotype. These basic RNase isoforms had a pI between 9.7 and 10.6. In a self-compatible mutant, no ribonuclease activity was observed in the basic region of the gel, indicating further support for the role of ribonucleases in self incompatibility.

Work in *Malus domestica*, another species which displays gametophytic self incompatibility, has demonstrated the specific inhibition of pollen germination and tube growth can be achieved *in vitro* using fractionated style protein extracts. Speranza and Calzoni (1990) showed that two style protein fractions obtained by ConA-Sepharose 4B column fractionation could introduce specific inhibition to the *in vitro* bioassay. These two fractions differed in that only one fraction displayed specific inhibition of pollen

germination while both fractions conferred specific inhibition of pollen tube growth.

Identification of self-incompatibility related glycoproteins (S_a, S_b, S_c, S_d, S_e, and S_f) in *Malus domestica* was done using two dimensional electrophoresis. Sassa et al. (1994) showed that style glycoproteins associated with self incompatibility could be resolved on a two dimensional polyacrylamide gel. These glycoproteins also cross reacted with antiserum raised against the S_4 glycoprotein of *Pyrus serotina*. Molecular weight determinations were estimated between 27 and 30 kD.

Isolation of cDNA clones for *Malus domestica* self-incompatibility alleles produced clones for the S_2 and S_3 alleles of the cultivar Golden Delicious (Broothaerts et al., 1995). Genomic DNA fragments representing part of the *S*-gene were amplified by PCR using degenerate primers. These degenerate primers were based on amino acid alignments of the known *S*-alleles of the *Solanacea* family. The amplified genomic fragments were used to screen a pistil specific cDNA library. Two pistil specific clones were identified, and analysis of their occurrence in a series of

apple varieties with defined *S*-phenotypes led to the conclusion that they corresponded to the S_2 and S_3 alleles.

Database searching now reveals that additional *Malus domestica* *S*-alleles have been isolated. The S_5, S_7, S_9, and a *S*-like sequences have been submitted to GenBank (Broothaerts et al., In press 1997).

Finally, this discussion should not be completely limited to self incompatible plant species. Stigmatic ribonucleases have been identified in the self compatible species *Prunus persica* (Roiz and Shoseyov, 1995). Experiments showed that the stigmatic ribonuclease was developmentally regulated, reaching maximum activity at the beginning of anthesis. Both the stigmatic ribonuclease and pancreatic ribonuclease A significantly inhibited pollen tube growth in an *in vitro* bioassay. The inhibitory effect of these ribonucleases could be eliminated by the addition of RNA as a competitive inhibitor in the bioassay. The significance of these findings is unclear, although it appears that the stigmatic ribonuclease plays a role in pollination.
In the self-compatible species *Arabadopsis thaliana*, three genes with homology to *S*-ribonucleases have been identified (Taylor and Green, 1991). These ribonucleases showed significant homology with known *S*-alleles. The functional

role of these Rnases in *Arabadopsis* organism is yet unknown, but their presence defines a broader class of plant ribonucleases.

Chapter 1

ANALYSIS OF SWEET CHERRY STYLAR PROTEINS

Introduction

Glycoproteins associated with *S*-alleles (*S*-glycoproteins) were first reported in sweet cherry (Mau et al., 1982; Williams et al., 1982). These isolated glycoproteins were shown by two dimensional electrophoresis to contain two components which were suggested to be the products of the two *S*-alleles. These proteins had approximate isoelectric points of 10.6.

S-allele proteins with similar properties were isolated from Solanaceous crops *Nicotiana alata, Petunia inflata, Solanum chacoense,* and *Lypcopersicon peruvanium* and were shown to encode ribonucleases (McClure et al., 1989, Singh et al., 1991, Xu et al., 1990, Chung et al., 1993).

In the *Rosaceae*, identification of six *S*-proteins by two dimensional electrophoresis in *Malus domestica* (Sassa et al., 1994) showed that these *S*-proteins had physical properties similiar to the *S*-proteins of Solanaceous crops

(M.W. ≅ 30,000 Da and basic pI). In Japanese pear, *S*-allele related basic RNases were identified in styles by isoelectric focusing (IEF) and staining for RNase activity (Sassa et al., 1992), again showing similiar physical properties to the Solanaceous species.

The objective was to determine if proteins with similar characteristics as the *S*-allele associated proteins could be identified in sweet cherry styles using genotypes differing in their *S*-alleles. If *S*-allele associated proteins were identified, a long term goal would be to obtain N-terminal sequence information for comparison with other *S*-allele sequences.

Materials and Methods

Plant material: Sweet cherry cultivars representing three different S-allele genotypes were used: Napoleon or Emperor Francis (S_3S_4), Ulster (S_2S_4), and Van (S_1S_3).

Protein extraction: Styles were collected from field grown trees when the flowers were in the balloon stage (one day prior to anthesis). This involved removing the ovary with a razor blade. PAGE analyses were performed on concentrated soluble stylar proteins. Approximately 20 styles per

cultivar were ground in liquid nitrogen. Then, 2 mls of 0.1 M Tris, 0.005 M EDTA , 0.014M B-mercaptoethnaol, and 2%(w/v) PVPP was added and the mixture was centrifuged at 30,000 g for 20 minutes. Proteins were then precipitated from the supernatant by the addition of acetone to a final concentration of 80%. The protein was resuspended in sample buffer (0.0675M tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and stored at -80° C or used immediately for electrophoresis.

Protein quantification: The amount of protein needed for PAGE analysis was determined empirically by loading and running varying amounts of protein extract on a PAGE gel followed by staining with 1% coomassie blue R-250.

SDS-PAGE: SDS-PAGE was performed as described by Laemmli (1970). The proteins were detected by staining with Coomassie blue. SDS-PAGE was subsequently performed according to Blank et al. (1982) using proteins where beta-mercaptoethanol was omitted from the extraction buffer and sample loading buffer as experiments (data not shown) showed that β-mercaptoethanol had an inhibitory effect on ribonuclease activity in the staining assays. The gels were stained for RNase activity using the procedure of Blank et

al. (1982) with the addition of 2 uM $ZnCl_2$ to the gel wash buffer (Christy Howard, per. comm.). For a detailed Rnase staining procedure, see Appendix A.

NEPHGE: Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) was performed on the pistil proteins of the cultivar Napoleon using the extraction procedure described above, except that the acetone precipitated proteins were resuspended in IEF loading buffer (O'Farrel et al., 1977). Gels were stained using a silver staining kit (Sigma Chemical Co., St. Louis, MO) and then dried.

Results

SDS-PAGE of stylar proteins of three cultivars are shown in Figure 1.1. The most abundant protein had a size of 29 kDa. The broad size of the 29 kDa band suggests that it may represent more than one protein with slightly different mobilites. Napoleon and Ulster, which share the S_4 allele, appear to have a component of the '29 kD band' that migrates faster than Van, which does not contain the S_4 allele.

RNase staining of Emperor Francis stylar proteins separated by SDS-PAGE identified 4 different regions of Rnase activity. The different areas resolved after different

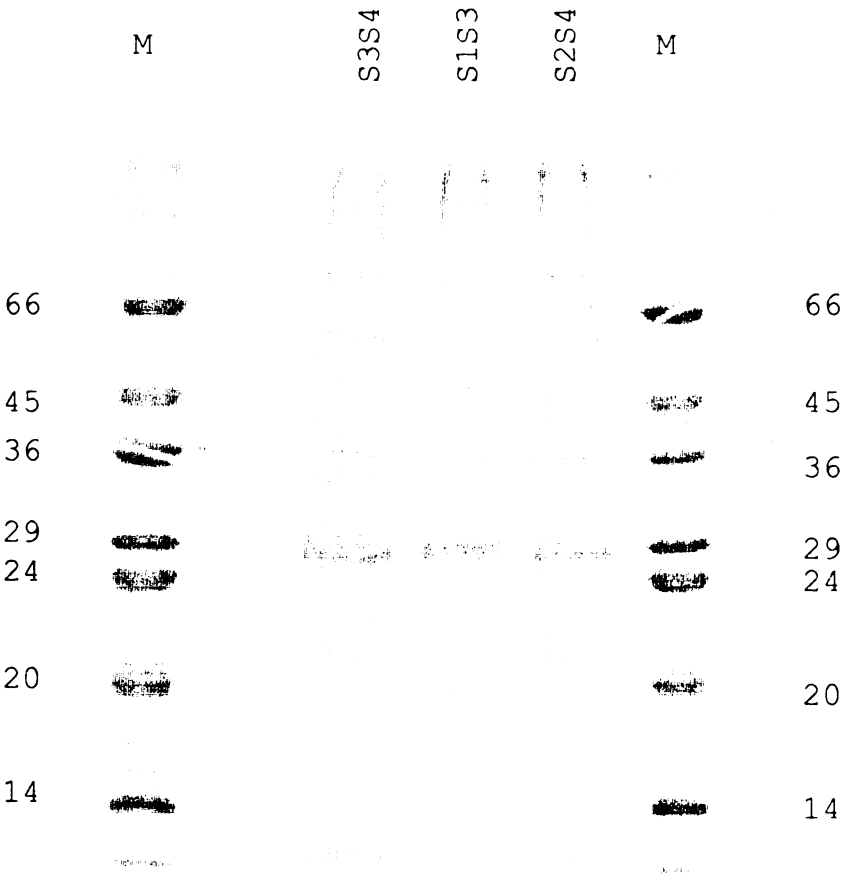


Figure 1.1 SDS-Page of Total Style Protein from the Cultivars Napoleon (S3S4), Van (S1S3), and Ulster (S2S4).

lengths of staining (from 10 minutes to 2 hours). The data is not shown due to low quality of the gel photographs.

Silver staining of two-dimensional NEPHGE gels of Napoleon stylar proteins identified two major basic proteins with pI ≅ 10.5 (Figure 1.2). The front of the isoelectric gradient is marked with a minus (-). The direction of migration during isoelectric focusing was from + to - . The second dimension was SDS-PAGE and was vertical with smaller proteins nearer the bottom of the gel. Hence, we see the two proteins are clearly seperated by size, with the smaller protein migrating nearer to the negative front. Of these two proteins, the larger protein looks to be more heterogenous, possibly consisting of two nearly identical proteins whose physical properties are so similiar that their separation was near the resolution limits of the gel.

Discussion

Examination of the proteins of the style by various electrophoretic techniques indicate that the *S*-proteins have very similar physical properties. Separation based solely on size (SDS-PAGE) was insufficient to clearly resolve the allelic forms of the proteins. The major proteins in the style were found to have a molecular weight of 29 kDa. In

earlier studies, two major antigenic glycoproteins were isolated and tested for their ability to inhibit pollen tube growth in vitro (Mau et al. 1982, Williams et al. 1982). The two stylar components, Antigen P and Antigen S, had molecular weights of 32 kD and 37-39 kD, respectively. Studies done by the mentioned investigators showed that Antigen S was able to inhibit *in vitro* growth of pollen tubes. My finding that the major protein of the style is29kD indicates one of two conclusions; that earlier studies failed to correctly identify the *S*-protein, or the *S*-protein is not the most abundant protein in sweet cherry styles.

Since the findings in Japanese pear and apple both show *S*-proteins to have a molecular weight between 27,000 and 30,000 Da, our estimates of 29,000 Da seem to be reasonable.

Further investigation of cherry stylar proteins by two-dimensional electrophoresis shows two candidate *S*-proteins fitting the description previously identified *S*-proteins (see literature review). Two proteins were found with a pI of greater than 10. Studies such as those done by Sassa et al. (1994) and Jahnen et al. (1989) in which stylar protein extracts from different *S*-genotypes were compared by 2 dimensional NEPHGE could provide the answer to the identity of the *S*-proteins in cherry.

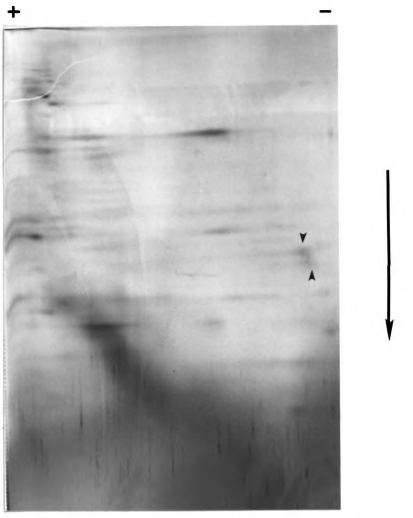


Figure 1.2 Two Dimensional NEPHGE of Stylar Proteins. Small arrows indicate candidate proteins fitting the description of previously identified *S*-proteins (see literature review for a complete description). The long arrow to the right of the gel indicates the direction of migration in the SDS dimension.

To investigate the possibility of the 29 kDa bands observed in Napoleon, Van, and Ulster being *S*-allele associate RNases, the following would have to be demonstrated: 1) they segregate with known *S*-allele phenotypes, 2) their N-terminal sequence has homology to known S-allele associated RNases, and 3) they display RNase activity.

RNase activity staining identified several isoforms in stylar extracts. It is unlikely this assay could identify genotype specific RNases due to the inability of SDS-PAGE experiments to resolve allele specific *S*-proteins. In future attempts to identify *S*-proteins, this assay would be useful when applied to purified candidate proteins. The presence of several ribonucleases in the style invites questions as to the function of the non-SI associated ribonucleases that could be the focus of future research.

During the time the electrophoresis experiments were being conducted, numerous publications were reported that concerned amino acid sequences of Rnases, in general, and *S*-allele associated stylar RNases, in particular. Based on this new information, we decided that a more efficient strategy to obtain an *S*-allele associated RNases in sweet cherry would be to utilize the conserved DNA sequences. This strategy is described in Chapter 2.

Chapter 2

ISOLATION OF A RIBONUCLEASE GENE FROM SWEET CHERRY

Introduction

Numerous *S*-allele associated RNases have been cloned from plants in the Solanaceous and Rosaceous families (see literature review for more detail). These RNases have been demonstrated to be temporally and spatially expressed at the site of *S*-allele activity. *In vivo* evidence that the *S*-allele proteins are involved in self-incompatibility was demonstrated in *Petunia inflata* (Lee et al., 1994) when the transgene encoding the S_3 protein was expressed in S_1S_2 plants and S_3 pollen was rejected in the styles of these transgenic plants.

Comparisons of the amino acid sequences reveal that the *S*-proteins are highly divergent with sequence identity between alleles from the same species (*N. alata*) ranging from 43.1% to 69.9%. However, there are also distinct conserved regions which have sequence similarity to two fungal

ribonucleases RNase T2 and RNase Rh (Khery-Pour et al., 1990).

The objective was to identify putative *S*-allele associated RNases from sweet cherry using DNA primers designed to amplify segments of genomic DNA or cDNA within the coding region of a ribonuclease. PCR primers were used which corresponded to amino acids conserved among the Solanaceae *S*-allele Rnases and fungal Rnases T2 and Rh. Template DNA was not only stylar cDNA, but also genomic DNA because sweet cherry styles are only available for a short period during sweet cherry bloom.

Materials and Methods

A flow chart of the strategy for obtaining RNase sequences from sweet cherry is presented in Figure 2.1. Amplified fragments obtained from this strategy were tested for their potential as *S*-allele associated RNases by Southern analysis using different genotypes, by differential expression (stylar vs. leaf), and by sequencing.

Plant material: Newly expanding leaves from the sweet cherry cultivar Emperor Francis (EF)(*S*-genotype S_3S_4) were harvested from greenhouse grown plants. The leaves were

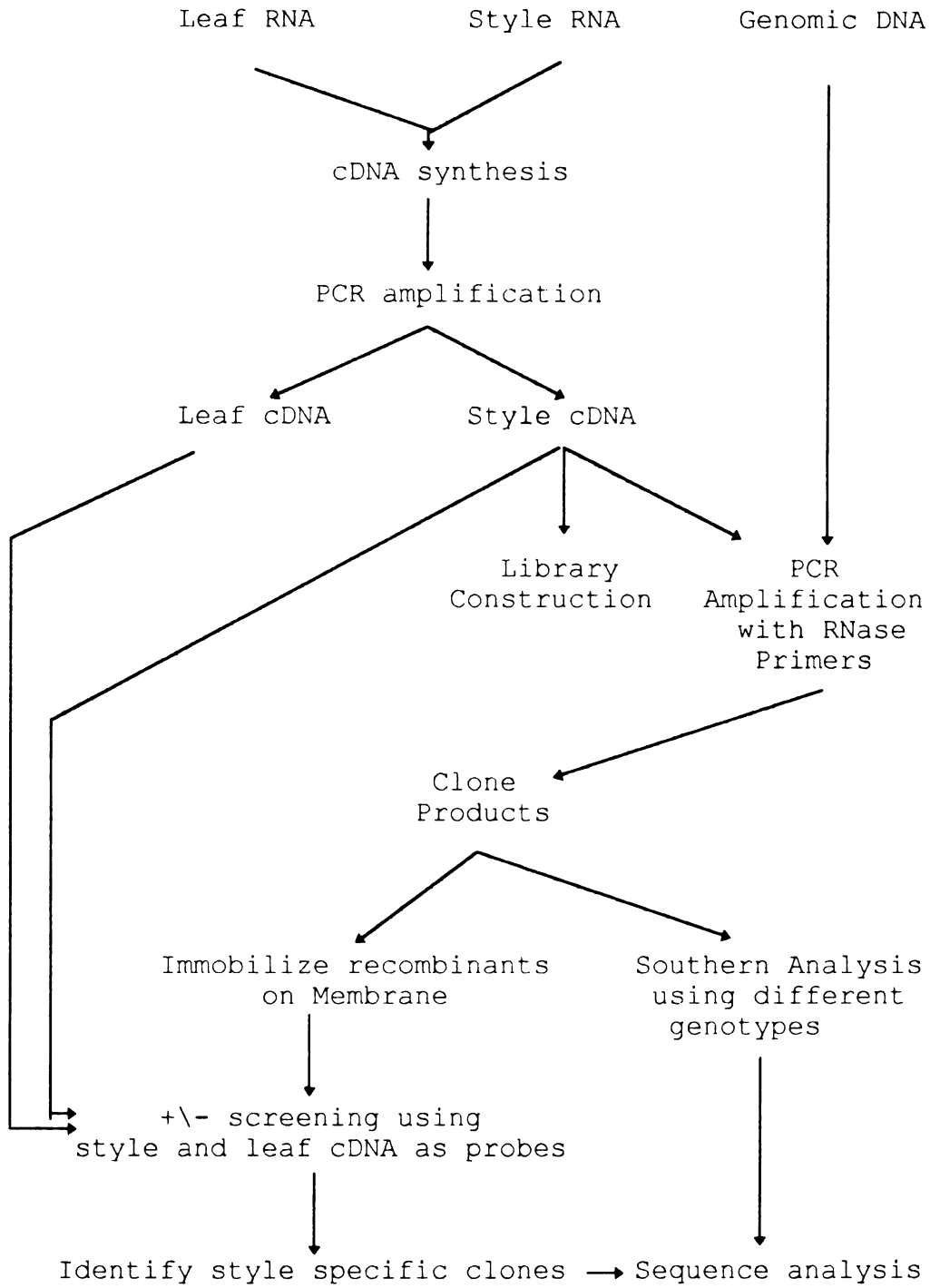


Figure 2.1 Overview of Methodology.

freeze dried and stored at -80° C for DNA isolations, or stored under liquid nitrogen without freeze drying for RNA extraction. Styles from EF were collected from branches that had been cut from field-grown trees one to two days prior to anthesis. Whole styles and stigmas (subsequently referred to as styles) were separated with a razor blade from the ovary of those flowers which were in the late balloon stage. The styles were immediately placed in liquid nitrogen until RNA extraction was performed.

The different sweet cherry genotypes used for the Southern analyses were the cultivars Van (S_1S_3), Ranier (S_1S_4), Napoleon (S_3S_4), Emperor Francis (S_3S_4) and Schmidt (S_2S_5). Leaves from these cultivars were collected in the spring from field grown trees. Leaves were collected, transported to the laboratory on dry ice, freeze dried, and stored at -20° C until DNA extraction was performed.

Nucleic Acid Isolations: DNA was isolated from the freeze dried leaves using the method of Stockinger et al. (1996) and checked on a 1% agarose gel in 0.5x TBE for degradation. RNA was isolated from approximately 1 gm of leaf and stylar tissue by the method of Manning (1991) with the following modifications. For stylar tissue, four phenol chloroform

isoamylalcohol (25:24:1) extractions were performed due to the high ribonuclease levels in the styles. For both leaf and stylar extractions, the [Na+] in the first butoxyethanol precipitation was adjusted to 100 mM. An extra differential precipitation was performed to further reduce the absorbance at 230 nm. RNA quality was checked by electrophoresis in a 1% agarose gel in 0.5x TBE. Purity of the RNA and DNA preparations were checked by absorbance readings at 230 nm, 260 nm, 280 nm and 310 nm.

cDNA synthesis: PCR amplified leaf and style cDNA was prepared according to Jepson et al. (1991) using a cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN). Two mg of total RNA was used in a cDNA reaction with dT_{15} primers. After second strand cDNA synthesis, EcoRI linkers were ligated to the size-selected cDNA (greater than 500bp), unligated linkers were removed using Microcon concentrators (Amicon, Beverly, MA). The cDNA was then subjected to 30 cycles of PCR using primers specific to the linkers.

Leaf cDNA was used as a probe on dot blots, while stylar cDNA was used as a probe on dot blots, as template in PCR amplification with RNase primers, and library construction.

Stylar cDNA library: Ligated stylar cDNA in lambda gt10 was packaged, transformed, and plated using the Packagene *in vitro* packaging system (Promega, Madison, WI) following the manufacture's protocol. The quality of the library was checked by hybridizing pRE12, a ribosomal clone which spans both the 18S and 25S rRNA coding regions (Delseny et al., 1983), to plaque lifts from three plates containing approximately 4,500 plaques per plate. Labeling of the probe was done with the Radprime labeling kit (Gibco BRL, Gaithersburg, MD) and hybridization conditions were as described in Appendix C. This library was intended to be used to obtain a full length clone of putative RNases identified following PCR amplification.

RNase primers: Primers AI-1 and AI-2 (Figure 2.2) were designed based on *S*-allele and RNases amino acid sequence homology (Figure 2.3) (T.H. Kao personal communication). The AI primers were synthesized at the MSU Macromolecular Structure Facility. A second set of primers, PG-30 and PG-31, also designed to amplify RNases, were obtained from P. Green (Taylor and Green, 1991)(Figure 2.2). For both primer sets, the approximate number of bases between and including the two primer pairs according to the sequence alignment in Figure 2 is 180 bp. The AI and PG primers were used to

Primer AI-1[a]

```
      V
      I   H   G   L   W   P
5'- ATN CAT GGN CTN TGG CC -3'
        C     T
```

Primer AI-2[b]

```
      S
      C   C   T   G   H   K
5'- CA ACA NGT NCC ATG TTT -3'
    GT G           G   C
```

Primer PG-30[c]

```
            H   G   L   W   P   D
5'- GAATTCAT GGN TTN TGG CCN GA -3'
    EcorI
```

Primer PG-31

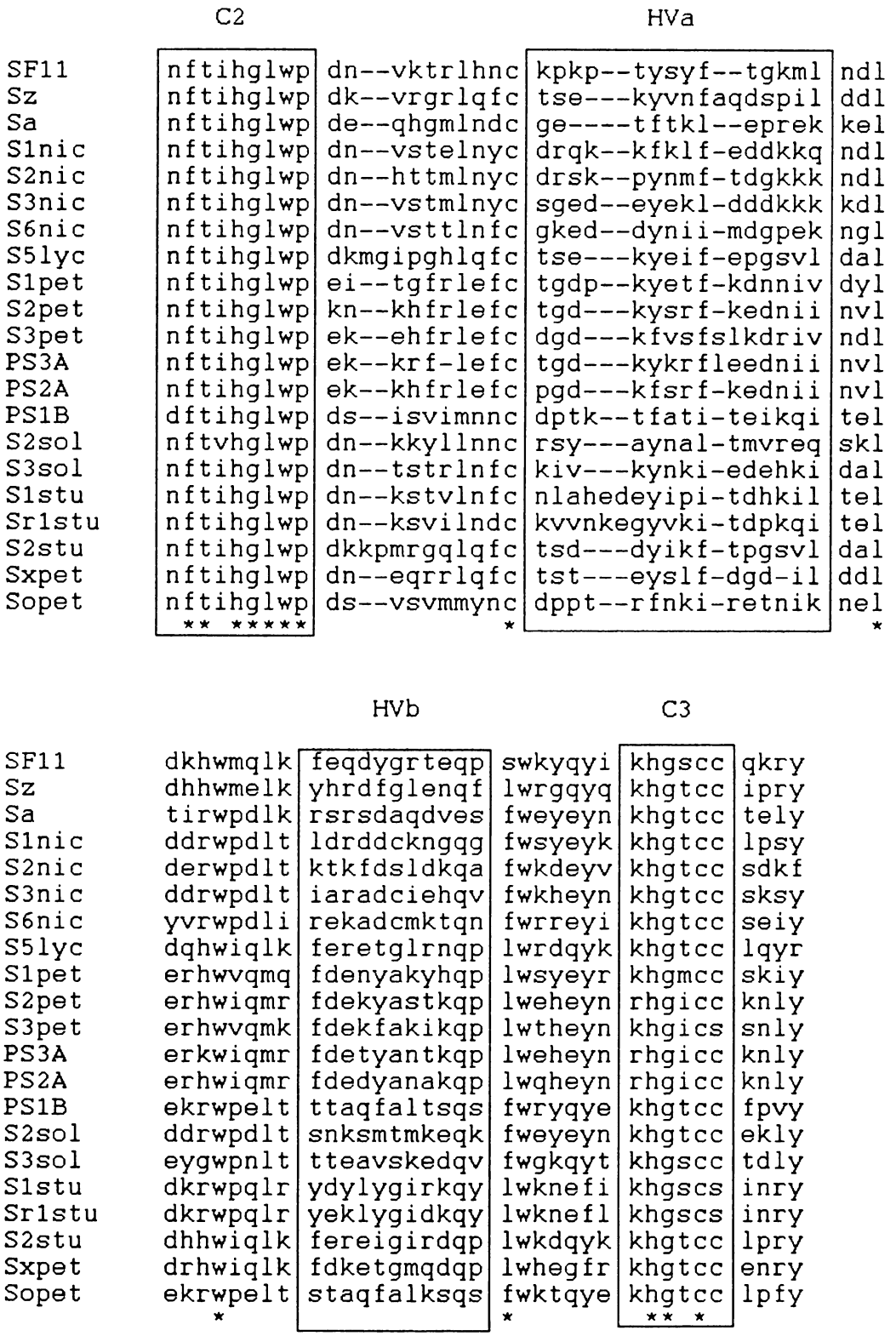
```
                          E
                          N
                          I   Y
        T   G   H   K     V   W   E
5'- CTCGAGT NCC ATG TTT TTT ATA TTC -3'
    XhoI        G   C   AAC GC  C
                        G   C
```

a - AI-1 and PG-30 represents the amino acids nearest the carboxy-terminus.
b - AI-2 and PG-31 represents the amino acid sequence near the N-terminus
c - PG primers are from Taylor and Green (1991).

Figure 2.2 PCR primers based on Rnase amino acid sequence homology.

Figure 2.3 Amino acid alignment of different *S*-allele Rnases. Amino acid alignments used to design the degenerate PCR primers. The alignments were kindly provided by T.H Kao. The * show completely conserved amino acids. The boxes highlight conserved regions C2 and C3, and hypervariable regions HVa and HVb (Kehyr-Pour et al., 1990).

Figure 2.3


C2
HVa
HVb
C3
SF11 nftihglwp dn--vktrlhnc kpkp--tysyf--tgkml ndl dkhwmqlk feqdygrteqp swkyqyi khgscc qkry
Sz nftihglwp dk--vrgrlqfc tse---kyvnfaqdspil ddl dhhwmelk yhrdfglenqf lwrgqyq khgtcc ipry
Sa nftihglwp de--qhgmlndc ge----tftkl--eprek kel tirwpdlk rsrsdaqdves fweyeyn khgtcc tely
S1nic nftihglwp dn--vstelnyc drqk--kfklf-eddkkq ndl ddrwpdlt ldrddckngqg fwsyeyk khgtcc lpsy
S2nic nftihglwp dn--httmlnyc drsk--pynmf-tdgkkk ndl derwpdlt ktkfdsldkqa fwkdeyv khgtcc sdkf
S3nic nftihglwp dn--vstmlnyc sged--eyekl-dddkkk kdl ddrwpdlt iaradciehqv fwkheyn khgtcc sksy
S6nic nftihglwp dn--vsttlnfc gked--dynii-mdgpek ngl yvrwpdli rekadcmktqn fwrreyi khgtcc seiy
S5lyc nftihglwp dkmgipghlqfc tse---kyeif-epgsvl dal dqhwiqlk feretglrnqp lwrdqyk khgtcc lqyr
S1pet nftihglwp ei--tgfrlefc tgdp--kyetf-kdnniv dyl erhwvqmq fdenyakyhqp lwsyeyr khgmcc skiy
S2pet nftihglwp kn--khfrlefc tgd---kysrf-kednii nvl erhwiqmr fdekyastkqp lweheyn rhgicc knly
S3pet nftihglwp ek--ehfrlefc dgd---kfvsfslkdriv ndl erhwvqmk fdekfakikqp lwtheyn khgics snly
PS3A nftihglwp ek--krf-lefc tgd---kykrfleednii nvl erkwiqmr fdetyantkqp lweheyn rhgicc knly
PS2A nftihglwp ek--khfrlefc pgd---kfsrf-kednii nvl erhwiqmr fdedyanakqp lwqheyn rhgicc knly
PS1B dftihglwp ds--isvimnnc dptk--tfati-teikqi tel ekrwpelt ttaqfaltsqs fwryqye khgtcc fpvy
S2sol nftvhglwp dn--kkyllnnc rsy---aynal-tmvreq skl ddrwpdlt snksmtmkeqk fweyeyn khgtcc ekly
S3sol nftihglwp dn--tstrlnfc kiv---kynki-edehki dal eygwpnlt tteavskedqv fwgkqyt khgscc tdly
S1stu nftihglwp dn--kstvlnfc nlahedeyipi-tdhkil tel dkrwpqlr ydylygirkqy lwknefi khgscs inry
Sr1stu nftihglwp dn--ksvilndc kvvnkegyvki-tdpkqi tel dkrwpqlr yeklygidkqy lwknefl khgscs inry
S2stu nftihglwp dkkpmrgqlqfc tsd---dyikf-tpgsvl dal dhhwiqlk fereigirdqp lwkdqyk khgtcc lpry
Sxpet nftihglwp dn--eqrrlqfc tst---eyslf-dgd-il ddl drhwiqlk fdketgmqdqp lwhegfr khgtcc enry
Sopet nftihglwp ds--vsvmmync dppt--rfnki-retnik nel ekrwpelt staqfalksqs fwktqye khgtcc lpfy

amplify stylar cDNA and genomic DNA,respectively.

Amplification and cloning of putative RNase sequences: Amplification of genomic DNA with the PG primers and stylar cDNA with the AI primers was done according to Taylor and Green (1991) with the following modification. The second PCR amplification from the gel isolated bands was identical to the first PCR amplification. PCR products were concentrated and the primers removed in a Microcon concentrator (Amicon, Beverly MA). PCR products from genomic DNA amplification were ligated into EcoRV cut pBluescript II KS-plasmid (Stratagene, LaJolla, CA) which was modified to contain a dT overhang (Marchuk et al., 1991). The major bands from stylar cDNA amplification were purified from a 5% acrylamide gel by cutting the ethidium bromide stained DNA from the gel and eluting the DNA by the crush and soak method of Sambrook et al. (1989). Five microliters of the resulting DNA was reamplified using the same PCR conditions. The reamplified products were treated with Proteinase K (Crowe et al., 1991), subjected to a T4 DNA polymerase fill in reaction (Sambrook et al., 1989), and cloned by blunt end ligation into HincIII digested, dephosphorylated pUC118. Ligation products were electro-transformed (BioRad electro-transformation and pulse controller instruction manual,

version 2-89) into electorcompetent DH5α *Escherichia coli* cells. Positive recombinants were selected by blue white selection in the presence of 5-bromo-4-chloro-3-indoyl-B-galactoside (X-gal) and isopropylthio-B-galactoside (IPTG)(Sambrook et al., 1989). Plasmids were then isolated from white colonies by the alkali lysis method (Sambrook et al., 1989) and digested with the appropriate restriction enzymes to examine insert size. Cloned PCR amplification products of EF leaf genomic DNA were designated pG (1-273). Cloned PCR amplification products of EF stylar cDNA were designated pS (1-169).

Screening of PCR Clones: Those clones which contained inserts were used for +/- dot blot screening (Berger and Kimmel, 1987). 750 ng of plasmid DNA was immobilized on the membrane using a dot blot manifold (Schleicher and Schuell, Keene, NH). The following clones were included as controls: pBS(-), pUC118, and an *Arabidopsis* tubulin sequence cloned into pBR322 (kindly provided by M. Thomashaw). Double stranded leaf and stylar cDNA obtained from adapter ligation/PCR (200 ng) were ^{32}P labeled using a random octamer labeling kit (Gibco BRL, Gaithersburg, MD) and separately hybridized to the same dot blots of the recombinant plasmids. Prehybridizations, hybridizations,

and washes were done according to established protocols (Appendix C).

Southern analysis: Genomic DNA from 5 different cultivars was isolated and digested with either HindIII or EcoRI (3 unit/ug). Ten micrograms of each digest were run on a 1% agarose gel at 22V for 18 hours and then transferred to a nylon membrane. Probes were PCR amplified using vector specific primers which flank the insert (Appendix B). The inserts were labeled with ^{32}P dCTP using the Radprime labeling kit (Gibco BRL, Gaithersburg, MD). Prehybridization and hybridization conditions were as previously described.

Clone sequencing: Plasmid DNA for sequencing was isolated using the Magic Wizard Miniprep kit (Promega, Madison, WI). Sequencing of selected cloned PCR products was done either at the MSU Sequencing Facility using an automated ABI 373 sequencer and dye-primer sequencing reactions, or using the Amplitaq Cycle Sequencing Kit using ^{32}P end-labeled sequencing primers (Perkin Elmer, Foster City, CA). All autoradiographs were scored twice. Analysis of the sequence data was done using the DNASIS software package. All DNA sequences were used as queries against the non-redundant sequence database GenBank using BLASTN (Altschul et al.,

1990). Amino acid homology searching was done using TBLASTN and (Altschul et al., 1990). Amino acid alignments were done using Sequence Navigator (ABI Applied Biosystems, Foster City, CA) using the clustal alignment algorithm.

Results

RNA isolations: The only previously published protocol for isolation of RNA from *P. avium* was published by Manning (1991). This protocol was used successfully with leaf tissue with a slight modification of the Na+ concentration in the extraction buffer. The quality was demonstrated by agarose gel electrophoresis and subsequent analysis of first strand cDNA synthesis from mRNA isolated by oligo dT cellulose chromatography. However, when this protocol was applied to stylar tissue, agarose gel electrophoresis revealed severe degradation of total RNA, which was presumably due to the high levels of ribonuclease activity in the style. Stylar tissue is high in ribonuclease activity as observed in the RNase activity gels described in Chapter 1 (data not shown).

Modifications of the leaf RNA extraction protocol were made following the protocol of Kheyr-Pour et al. (1990). In short, this involved doing 4-5 phenol extractions to ensure

that all of the protein was removed before proceeding with the butoxyethanol precipitations (Table 2.1). Typical ratios of absorbance reading for 260nm/230nm and 260nm/280nm were greater than 2.0 and 1.8, respectively.

Table 2.1 - Absorbance readings for stylar RNA isolations.

	A230	A260	A280	conc. in mg/ml	260/230	260/280
Procedure 1[a]	1.343	0.836	0.552	3.346	0.623	1.515
	1.322	0.941	0.609	3.762	0.711	1.546
	2.145	1.556	1.030	6.226	0.725	1.510
	1.495	0.724	0.504	2.896	0.484	1.436
	0.978	0.932	0.509	3.730	0.953	1.834
	1.546	1.521	0.820	6.086	0.984	1.856
Procedure 2[b]	0.412	0.699	0.381	2.800	1.700	1.830
	0.386	0.650	0.358	2.600	1.680	1.820
	0.255	0.536	0.286	2.142	2.100	1.870
	0.099	0.217	0.114	0.866	2.190	1.900
	0.040	0.104	0.054	0.834	2.610	1.930
	0.093	0.213	0.123	1.704	2.290	1.730

a Procedure 1 represents data collected before modification of the protocol.
b Procedure 2 represents data collected after modification of the isolation protocol to contain 2 to 3 extra phenol chloroform extractions and one extra 2-butxyethanol precipitation.

cDNA synthesis and cloning: Yield of first strand cDNA synthesis was calculated measuring total and incorporated radioactivity by scintillation counting (see Appendix D for derivation of formulas). For the control reaction, 154.5 ng of cDNA was made using the mRNA supplied by the manufacturer. The resulting yield was 30.9%, which is the expected yield from mRNA according to the manufacturer. For the cDNA reaction with stylar RNA, the yield was 70.9 ng.

Percent yield was not calculated because total RNA (15 ug) was used instead of mRNA.

Quality screening of the stylar cDNA library: To check for the possibility of chloroplast and ribosomal DNA contamination in the lambda gt10 stylar cDNA library, plaque lifts were done and hybridized with the rDNA probe pRE12, which contained the 18S and 25S coding regions (Delseny et al., 1983) and a chloroplast DNA probe pB81 (kindly provided by B. Sears). Lifts were made from 3 plates containing a total of approximately 5250 plaques. Fifty-two positive signals were identified following hybridization with pRE12. Therefore, the library appears to have 1% rDNA sequence. Nineteen positive signals were obtained with pB81. Assuming that the library contains random chloroplast sequences and because the pB81 clone represents about 10% of the chloroplast genome, the library appears to contain about 4% chloroplast sequences.

Ten randomly selected clones from the library had the following insert sizes (bp): 615, 490, 420, 400, 380, 370, 280, and 250. The average insert length (n=10) was 390 bp.

Table 2.2 - Nomenclature and origin of pS clones

	Transformation Number[a]	Size in bp	Number of Recombinants	pS Numbers
	1	210	0	n/a
	2	180	85	86-170
	3	250	48	1-32, 57-72
	4	310	37	33-56, 73-85
Totals	4	180-310	170	pS(1-170)

a - Transformation numbers refer to which PCR band was cut from the gel and reamplified for cloning. These numbers only apply to the cloning of amplified products of stylar cDNA.

PCR amplification of stylar cDNA with the AI primers and cloning of products: Stylar cDNA not cloned into lambda gt10 was used in PCR reactions with the AI-1 and AI-2 degenerate primers. Four major bands were evident on a 5% non-denaturing polyacrylaminde gel. The molecular weights of the four major bands were 210 bp, 180 bp, 250 bp, and 310 bp. These were identified as band 1-4, respectively. Each of the four bands was isolated from the gel, re-amplified, and re-analyzed on an agarose gel. The resulting products were not homogeneous, rather they contained the major band of interest and usually a slightly smaller band. These DNAs (all of the bands from the second PCR) were cloned into pUC118 and identified by transformation numbers 1-4, which correlated with the specific band which was cloned. The number of recombinat plasmids per ligation ranged from zero to 85 (Table 2.2). All of the 170 recombinant plasmids were then isolated and digested with the appropriate restriction

enzymes to measure insert size via agarose gel electrophoresis. The most common band identified by plasmid digestion was 185pb, 250bp, and 305bp for transformations 2, 3, and 4, respectively (transformation 1 yielded no positive transformants). These sizes were in agreement with the size of the PCR product prior to cloning.

PCR amplification of EF genomic DNA with the PG primers and cloning of products: PCR amplification of EF genomic DNA using the degenerate primers PG-30 and PG-31 yielded six distinct bands on a 1.4% agarose gel with molecular weights of approximately 270 bp, 420 bp, 550 bp, 590 bp, 680 bp and 980 bp. Following gel isolation and cloning procedures, recombinant plasmid DNA was isolated from a total of 350 white colonies. Verification of insert was checked by running the plasmid preps on a 1.2% agarose gel. Those plasmids (6 out of 350) which appeared to have an insert were further investigated by restriction enzyme digestion. Of the inserts examined, pG29, pG47, pG115, and pG207 had insert sizes of 270, 420, 700, 420, which was concordant with the observed sizes of the PCR products. A fifth clone chosen for analysis, pG293, had an insert size of 230 bp.

The sixth clone, pG110, was also investigated further by

southern analysis. Isolated plasmid DNA from pG110 displayed patterns of supercoiling consistent with a recombinant pBluescript plasmid. When the pG110 plasmid DNA was digested with EcoRI or double digested with EcoRI and HindIII, the digestion patterns were the same, indicative of the absence of a known HindIII site. Therefore, I was not able to accurately determine the size of the insert contained in pG110 using these enzymes. Estimation of the pG110 insert was possible by examining the results of a EcoRI digestion. EcoRI digestion indicates a linear size of the recombinant plasmid to be about 6000 bp. Thus, by subtraction of the known size of pBluescript, it can be inferred that the size of the insert is about 3000 bp (this is a crude estimate and could vary by as much as ± 500 bp).

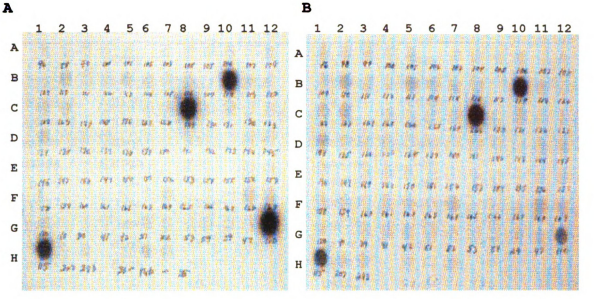
Dot blot screening of pS and pG clones: Recombinant plasmid of 6 pG clones and 163 pS clones were hybridized to stylar cDNA. Six clones gave high signals and an additional eight gave medium signals (Table 2.3, Figure 2.4). Since the probe was cDNA and not total genomic DNA, signal is not proportional to genomic copy number. The dot blot membrane was then stripped and exposed to film for 7 days to ensure that the previous probe had been completely stripped from the membrane prior to hybridization with leaf cDNA.

Table 2.3 - Copy Number Indicated by Dot Blot Hybridization of Labeled Stylar cDNA to pS and pG Clones.

High Copy Signals	Medium Copy Signals	Low Copy Signals
pS25	pS13	Remaining 155
pS95	pS56	pS and pG clones.
pS118	pS110	
pS129	pS113	
pG110	pS123	
pG115	pS134	
	pS164	
	pS168	

When the dot blots were hybridized to leaf cDNA, 6 clones gave high signals and 8 clones gave medium signals. When the two autoradiographs were compared, no style specific clones were identified (see Figure 2.4 for an example).

Southern analysis: Five pG clones and 27 pS clones were chosen for use in the Southern analysis with the different sweet cherry *S*-allele genotypes (Table 2.4, Figures 2.5 and 2.6). The pG207 clone was not used because preliminary sequencing information had shown that it was identical to pG47. Choosing which pS clones to use was based on size. Different size representatives from each of the three transformations were used.



In A, stylar cDNA was used as the labeled probe. In part B, leaf cDNA was used as the labeled probe. Wells A1-G9 are pS clones, wells G10-H3 are pG clones, and wells H5-H8 are controls, pBS(-), tubulin gene, pUC118, and TE buffer respectively. Detailed layout of this figure can be found in Appendix 4.

Figure 2.4 - Dot Blot Analysis of pS and pG Clones.

Only pG110 displayed an *S*-allele genotypic specific pattern (Figure 2.5 C). A band in both the HindIII digest and on the EcoRI digest was present exclusively in the three cultivars with an S_3 allele. pG110 had a low copy number, as would be expected for an *S*-allele, based on signal intensity on the Southern membrane. Two groups of clones gave identical hybridization patterns suggesting that they might be duplicate clones.

Table 2.4 - Clones Used as Probes Against Southern Blots of Different S-Genotypes.

Probe	Copy Number[a]	Bands	Group[b]	Probe	Copy Number[a]	Bands	Group[b]
Lambda 1	High	<5	3	ps056	Medium	>5	
Lambda 2	High	<5	3	ps085	Low	<5	
Lambda 3	High	<5		ps103	Low	<5	1
Lambda 4	High	<5		ps116	Low	<5	1
pg29	Low	<5		ps118	Low	<5	
pg47	Low	<5		ps123	Medium	<5	
pg110	Low	>5		ps134	Low	<5	
pg115	High	<5		ps140	Low	<5	1
pg293	High	>5		ps141	?	<5	
ps001	Low	<5		ps142	Low	<5	1
ps008	Medium	<5		ps143	Low	<5	1
ps009	Low	<5		ps145	Low	<5	1
ps013	Medium	<5		ps149	Medium	>5	
ps023	Low	<5		ps150	Low	<5	1
ps027	Low	<5	2	ps155	Low	<5	1
ps028	Low	<5	2	ps161	Low	<5	1
ps033	Low	<5		ps164	Medium	>5	
ps041	Low	<5		ps168	Low	<5	

a - Copy number is based on signal intensity on the membrane.
b - Group refers to those clones which gave identical hybridization patterns.

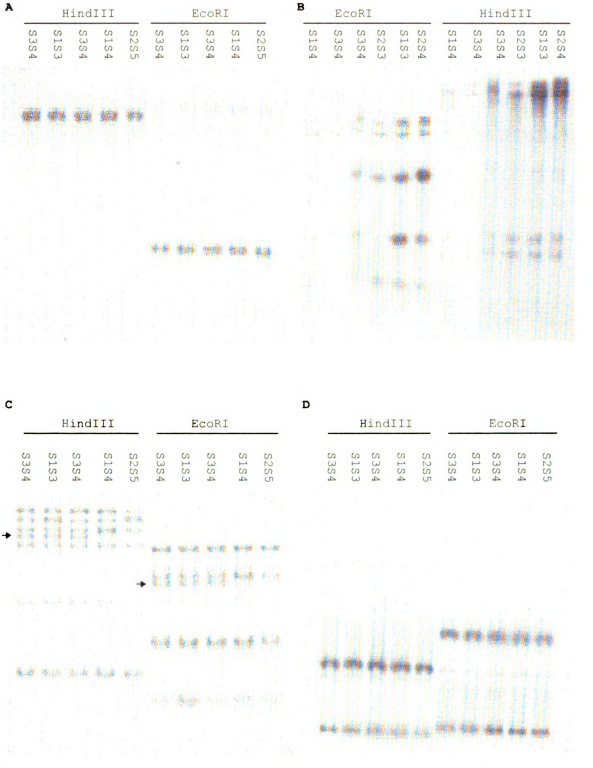


Figure 2.5 - Autoradiographs of pG Clones 29, 47, 110, and 115 (A-D respectively). Arrows on C (pG110) indicate the S_3 specific band.

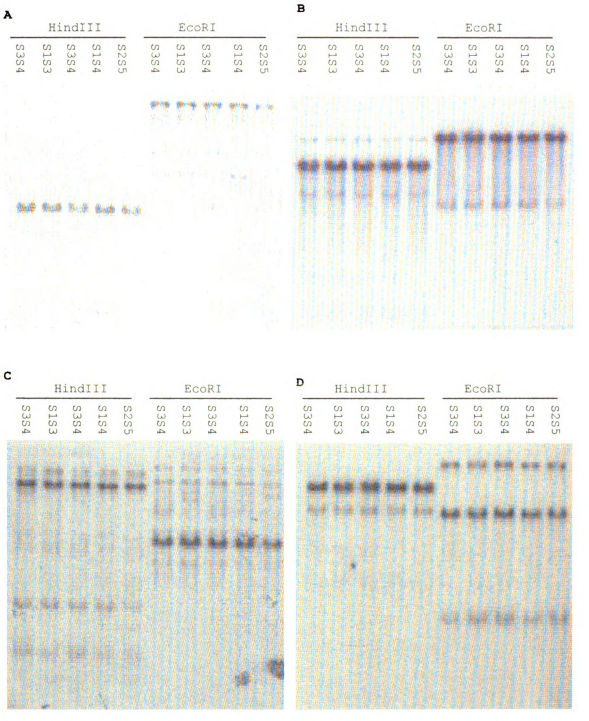


Figure 2.6 - Autoradiographs of pS Clones 1, 8, 9, 23, 27, 103, 141, and 149 (A-H respectively - see next page for E-H).

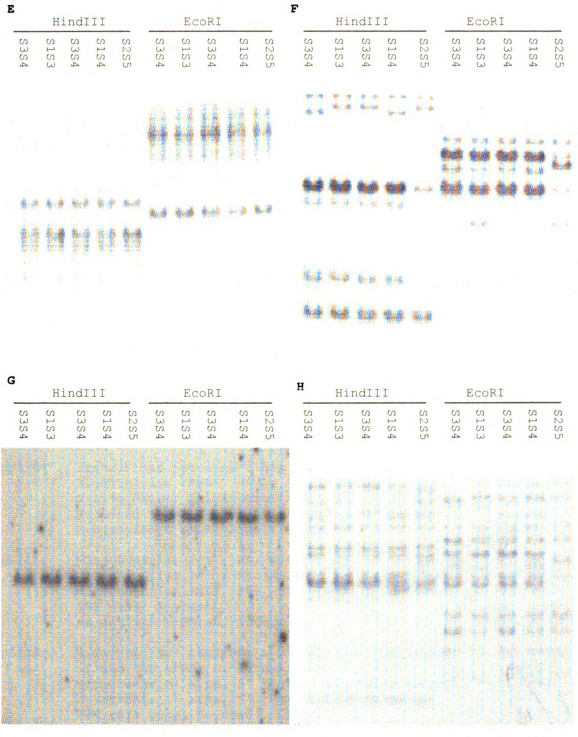


Figure 2.6 - Autoradiographs of pS Clones 1, 8, 9, 23, 27, 103, 141, and 149 (A-H respectively).

Sequencing of the pS and pG clones: Five of the six pG clones and four of the low copy pS clones were sequenced to see if they had homology to known ribonucleases. pG110 was not sequenced due to the size of the insert and the inability to reproduce the Southern analysis results.

Only two of the 10 clones sequenced had significant homology with sequences in the non-redundant Genbank sequence database when searching using BLASTN (Table 2.5). pG115 likely contains an 18S rDNA sequence. This is in agreement with the results from the Southern analysis that pG115 is

Table 2.5 - Summary of BLASTN on all sequenced clones.

Clone	Sequence producing Highest-scoring Segment Pairs[a]	P(N)[b]
pS118f[c]	Transcribed sequence-clone YAPS01; A. thaliana	7.8 e-05
	Ribonuclease (RNS1) mRNA, A. thaliana	9.8 e-05
	S-like RNase; Malus domestica	0.00044
	Wounding-induced ribonuclease; Zinnia elegans	0.0038
	Ribonuclease mRNA; Zinnia elegans	0.099
pG115r	Mitochondrial 18S rRNA; Wheat	4.1 e-70
	Mitochondrial 18S rRNA; Maize	4.2 e-70
	Mitochondrion fMET, 18s, 5S repeat; T. aestivum	6.0 e-70
	18S rRNA; Soybean	6.7 e-70
	5S and 18S rRNA; L. luteus	1.2 e-69
pG115f	Mitochondrial 18S rRNA & 5S rRNA; O. berteriana	1.1 e-06
	5S and 18S rRNA; L. luteus	0.00012
	Mitochondrial atp6, 5' region; Nicotiana tabacum	0.00086
	Mitochondrial atpA, 5' region; O. berteriana	0.001
	18S rRNA gene; Soybean	0.0017
pG207r	Ig germline kappa b5 chain, JC-region; Rabbit	0.48

a - Databases searched were Non-redundant GenBank, EMBL, DDBJ, and PDB.
b - The first 5 hits in the database which had a Smallest Sum Probability of less than 0.5 are listed.
c - The suffix f or r on the clone name refers to the forward or reverse sequencing reaction.

represented in high copy in the cherry genome (Table 2.4). pS118 had significant homology to known RNase sequences which prompted us to resequence the clone in both the forward and reverse directions. Table 2.6 shows the new P-values and database hits for pS118 based on 420 and 475 bases of good sequence data in two separate forward sequencing reactions and 410 bases of good sequence data in a reverse sequencing reaction.

Table 2.6 - BLASTN search on pS118 second pass sequence information.

Sequences producing High-scoring Segment Pairs[a]	P(N)[b]
Lycopersicon esculentum mRNA for ribonuclease le	1.5 e-14
Zinnia elegans ribonuclease mRNA	1.1 e-13
Nicotiana alata RNase NE mRNA	1.8 e-11
Malus domestica S-like RNase gene	0.00056
Arabidopsis thaliana ribonuclease (RNS1) mRNA	0.013
Zinnia elegans wounding induced ribonuclease mRNA	0.088

a - Databases searched were Non-redundant GenBank, EMBL, DDBJ, and PDB sequences.
b - Hits with P(N) > 0.05 are not shown.

Identification of the AI primer sequences indicated that pS118 was chimeric. The sequence for the AI-1 primer started at position 316 with one base missing. The bases (AAGCACGGGACT) immediately before the AI-1 primer sequence represent complementary sequence to the primer AI-2. Therefore, pS118 resulted from the ligation of two insert DNAs prior to ligation into the vector. The first base of the AI-1 primer and the 5' end of the AI-2 primer is not

present due to treatment of the PCR products with T4 DNA polymerase prior to ligation.

The pS118 region of nucleotide homology with known RNases extended from position 321 of the insert to position 414 (for complete sequence of the chimeric insert of pS118, see Appendix F). Full sequence information for the RNase sequence of the insert is presented in Figure 2.7. The missing cytosine in the AI primer sequence is identified. Internal primer sequences were identified which could be used to amplify to RNase portion of pS118 for future experiments.

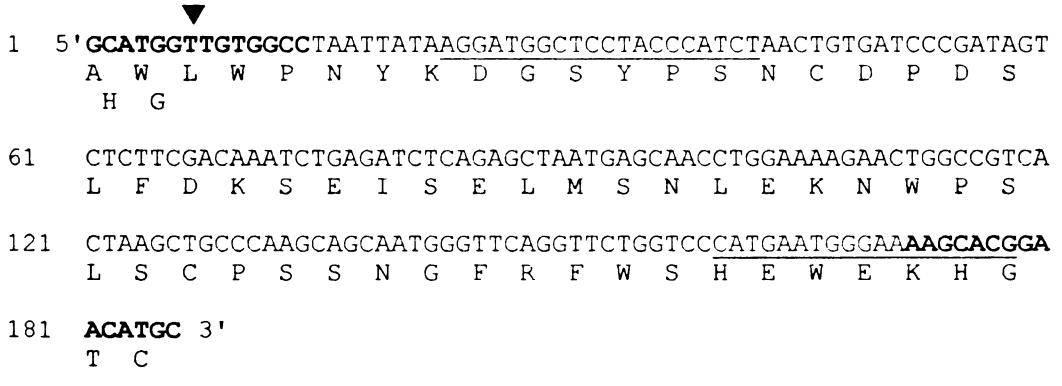


Figure 2.7 - Nucleotide and amino acid sequence of the ribonuclease portion of pS118. Bold sequence represents degenerate AI-1 and AI-2 primer sequences. Underlined sequence represents internal primers to amplify pS118 DNA for probes in Southern and Northern experiments. Asterisks represent internal conserved amino-acid sequence with 22 known S-alleles and 2 fungal ribonucleases. The ▼ marks the spot where a cytosine residue is missing when compared to primer sequence.

The BLASTX search of amino acid homology with the pS118 supported the conclusion that pS118 contains an RNase sequence (Table 2.7). The results presented in Table 2.7 were obtained without correcting for the missing base in the AI-1 primer region. The missing base caused two conserved amino acids (H and G) to be shifted out of reading frame; therefore, these two conserved amino acids did not contribute to the P(N) values. Amino acid sequence alingment with known plant ribonucleases revealed 13 conserved amino acid residues (Figure 2.8) when the sequence is corrected for the missing base in the AI-1 primer region.

Discussion

Isolation of intact mRNA was successful from sweet cherry leaf tissue as demonstrated by first stand leaf cDNA synthesis. However, RNA isolation from stylar tissue was problematic presumably due to the abundance of RNases present in the style (see Chapter 1). It is likely that extremely high quality RNA was not obtained from stylar tissue in the course of the project. It seems that the 2-butoxyethanol precipitations are required for the removal of polysaccarides from nucleic acids. The use of a guanidium based extraction buffer to eliminate RNase activity during the course of RNA isolation (Manning, 1991) was not

Table 2.7 - BLASTX Search on pS118 Second Pass Sequence Information.

Sequences producing High-scoring Segment Pairs[a]	P(N)[b]
Ribonuclease LE (RNase LE); starvation induced; tomato	4.6 e-27
Ribonuclease LX (RNase LX); starvation induced; tomato	5.5 e-27
Ribonuclease 3 precursor; Arabadopsis thaliana	1.2 e-26
Ribonuclease; Solanum lycopersicum	1.5 e-26
Ribonuclease; Lycopersicon esculentum	1.7 e-26
S-like RNase; Malus domestica	1.7 e-26
RNase NE; Nicotiana alata	1.9 e-25
Ribonuclease 1 precursor; Arabadopsis thaliana	2.6 e-25
Ribonuclease; Zinnia elegans	1.4 e-23
Ribonuclease; wounding induced; Zinnia elegans	1.5 e-23
Extracellular ribonuclease; Arabidopsis thaliana	3.6 e-18
S-like ribonuclease; Arabidopsis thaliana	5.6 e-18
Ribonuclease 2 precursor; Arabidopsis thaliana	1.2 e-17
Storage protein; Nelumbo nucifera	8.0 e-13
Ribonuclease TRV; Trichoderma viride	1.3 e-09
Ribonuclease T2; Aspergillus oryzae	2.0 e-09
Ribonuclease M; Aspergillus phoenicis	5.0 e-09
Ribonuclease; Momordica charantia	1.5 e-08
Ribonuclease; Physarum polycephalum	1.8 e-08
Ribonuclease; Oyster	1.0 e-07
Ribonuclease X25; Drosophila melanogaster	3.9 e-06
RNase Rh precursor; Rhizopus niveus	5.9 e-06
SI glycoprotein (allele S6); Lycopersicon peruvianum	5.6 e-05
S-allele-associated protein So precursor; Petunis hybrida	6.2 e-05
Ribonuclease LE2; Lentinus edodes	0.00018
S-13 RNase; Lycopersicon peruvianum	0.0011
Self-incompatibility ribonuclease; Solanum carolinense	0.0028
SI glycoprotein (allele S7); Lycopersicon peruvianum	0.0063
S-RNase; Physalis crassifolia	0.0071
Self-incompatibility gene S3; Nicotiana alata	0.014
S7-RNase; Malus domestica	0.029
S3-RNase precursor; Malus domestica	0.03
S2-Rnase precursor; Malus domestica	0.046
S11a-Rnase; Lycopersicon esculentum	0.087
Ribonuclease; Luffa cylindrica	0.096
S2-protein; Solanum chacoense	0.14
Ribonuclease; Lycopersicon esculentum	0.15
Self-incompatibility ribonuclease; Solanum carolinense	0.15
S9-RNase; Malus domestica	0.16

a - Databases searched were non-redundant GenBank translations, SwissProt, SwissProt update, PDB, and PIR databases.
b - Hits with probabilities greater than 0.2 are not listed for brevity.

```
Pa RNS1       HGLWPNYKDGSYPSNCDPDSVFDKSEISELMSNLEKNWPSLSC--PSS-NGFR--FWS

Md S2         hglwpsnmnrselfncsssnvtyakiq-nirtqlemiwpnvfnrknhl------gfwn
Md S3         hglwpsnvngsdpkkckttilnpqti-tnltaqleiiwpnvlnrkaha------rfwr
Md S5         hglwpsnfngpdpenckvkptasqtidtslkpqleiiwpnvfnradhe------sfwq
Md S7         hglwpsdsnghdpvncskstvdaqkl-gnlttqleiiwpnvynrtdhi------sfwd
Md S9         hglwpsnssgndpiyckntmnstki-anltarleiiwpnvldrtdhi------tfwn
Ph S1         hglwpdsisvimn-ncdptktfatiteikqitelekrwpeltttaqfaltsq--sfwr
Lp S13        hgvwpdhtsfvmy-dcdplkkyktiddtnilteldarwpqltstkiiglqfq--rfwe
Sp S7         hgvwpdhtdyimy-dcnpnkefkkiydkhllnklesrwpqltsheyaglndq--tfwk
RNASE LE      hglwpnnndgtypsncdpnspydqsqisdlissmqqnwptlac--psg-sgst--fws
RNSLX         hglwpnykdgkwpqncdressldesefsdlistmeknwpslac--pss-dglk--fws
At RNS1       hglwpnykdgtypsncdaskpfdsstisdlltsmkkswptlac--psg-sgea--fwe
At RNS2       hglwpdyndgswpsccyr-sdfkekeistlmdglekywpslscgspsscnggkgsfwg
At RNS3       hglwpnyktggwpqncnpdsrfddlrvsdlmsdlqrewptlsc--psn-dgmk--fwt
Ph S0         hglwpdsvsvmmy-ncdpptrfnkiretniknelekrwpeltstaqfalksq--sfwk
Conserved     HG*WP**********C**********************WP****************FW*

Pa RNS1       HEWEKHGTC
Md S2         rewnkhgac
Md S3         kqwrkhgtc
Md S5         kqwdkhgtc
Md S7         kqwnkhgtc
Md S9         kqwnkhgsc
Ph S1         yqyekhgtc
Lp S13        yeyrkhgtc
Sp S7         yeyekhglc
RNASE LE      hewekhgtc
RNSLX         hewlkhgtc
At RNS1       hewekhgtc
At RNS2       hewekhgtc
At RNS3       hewekhgtc
Ph S0         yqyekhgtc
Conserved     ****KHG*C
```

Md = *Malus domestica*, Ph = *Petunia hybridia*, Lp, LE, and LX = *Lycopersicon esculentum*, Sp = *Solanum peruvianum*, and At = *Arabadopsis thaliana*.

Figure 2.8 - Amino Acid Alignment of pS118 with Published Plant Ribonucleases. DNA sequence is corrected for missing base in the primer sequence.

investigated because of concern about reduced yield. Yield was an important consideration because styles could only be collected once a year and because of the number of styles required to obtain a sufficient quantity of RNA.

Plaque lifts done on the PCR amplified stylar cDNA library indicated the presence of ribosomal and chloroplast sequences. Apparently, these sequences were not completely removed prior to cDNA synthesis. DNase treatment prior to cDNA synthesis should have been done to eliminate contaminating chloroplast and nuclear DNA. The ribosomal sequences may have resulted from self priming of ribosomal RNA during the cDNA synthesis, which is a possiblity due to the nature of rRNA secondary structure, coupled with partial degredation or nicking of the rRNA (M. Thomashaw, pers. com.).

Due to the limitations in the quantity of stylar RNA and the resulting cDNA, the cDNA was amplified by PCR following the addition of linker sequences. Although this work and other published reports (Akowitz and Manuelidis, 1989, Belyavsky et al., 1989, Jepson et al., 1991) used this technique with total RNA, this step contributed to the presence of ribosomal sequences in the cDNA. If possible, purified mRNA

would be preferred.

A lambda gt10 cDNA library was constructed for the purpose of identifying full-length cDNA *S*-allele clones. This of course, has not yet been done, but it was useful to construct the library at the same time that cDNA was being generated for use in other experiments.

Dot blot experiments on the plasmid clones containing PCR generated inserts using the degenerate primers produced two classes of clones. The presence of a strong hybridization signal would indicate that the insert cloned in the plasmid exists in a high proportion in the cDNA population. This could be accounted for by three reasons: a) The sequence represents an abundant mRNA in the tissue from which the cDNA was made. b) The sequence represents a contaminating sequence which was present in high proportions prior to PCR amplification of the cDNA. c) The sequence represents some sequence that was preferentially amplified during PCR amplification of the cDNA.

The dot blot experiments hinged on the successful isolation of quality mRNA. This manifests in the reliability of the dot blot experiments in which tissue specific expression of

the pG and pS clones were assessed (because PCR was used to generate the pS clones, it is imaginable that all one would need is a few good mRNAs leading to a few good cDNAs, hence, high quality RNA may not have been so critical in the generation of the pS clones). The ramification of this may be that the dot blot experiments may not be valid even though the pS clones could contain expressed sequences.

Southern analysis using the pG and pS clones, on the other hand, was not dependent on any questions of RNA quality. Insight to the nature of the cloned DNA, such as copy number and polymorphism type, supports using the degenerate primers on genomic DNA as a viable strategy.

Of particular interest was pG110, because hybridization patterns on different incompatibility genotypes showed that this probe detected a band that was unique to three cultivars, all of which show the S3 phenotype. Unfortunately, this probe hybridized to more than one region This probe could be chimeric, a combination of two different inserts, which ligated together before ligation into the plasmid, as was the case with pS118. Ongoing work will result in the necessary sequence information to determine if pG110 contains ribonuclease sequence.

Investigation of pS clones by Southern hybridization experiments indicated that most of the clones represent low copy sequences. Since these clones were generated from the amplified style cDNA, we can be fairly confident that there exists a number of single copy sequences in the style cDNA pool. This was also demonstrated by the dot blot experiments in that only 4 out of the 81 pS clones tested in Figure 2.4 indicate a copy number other than low. While the dot blots were successful in indicating copy number with respect to the style cDNA pool, the Southern hybridization experiments gave an indication of uniqueness of each clone with respect to the population of pS clones obtained. The data collected and displayed in Table 2.4 show that many of the clones are unique. There does exist a group of clones which all give the same hybridization pattern, although these clones also show low copy within the cDNA pool. None of the pS clones tested showed *S*-genotype specificity. While it is possible that the enzymes used do not give a polymorphism at the incompatibility locus, I believe this to be unlikely in light of the nature of the locus, itself being highly polymorphic. The number of clones generated in the pS cloning experiment (172) made Southern analysis unfeasable as a method to test all clones.

Sequencing generates by far the most data about each individual clone. The reasoning behind choosing which clones to sequence was an attempt to select unique clones representing different cloning experiments. Database searching provided convincing evidence that pG115 contains 18S rDNA and that pS118 contains RNase sequence. Further evidence is available for both clones which support the database search results, mainly that Southern analysis shows that, based on signal intensity, pG115 is present with high copy number in the Cherry genome and that pS118 is present with low copy number. It was unfortunate that pS118 Southern analysis experiments did not yield sufficient resolution to determine if there was genotype specificity. Recent work by D.C. Wang (Unpublished data) has shown by Southern hybridization that the RNase portion of pS118 does not display genotype specificity and is single copy in the cherry genome.

Sequencing results show that pS118 is a chimeric clone. This was most likely the result of the insert-to-vector ratio in the ligation reaction (3:1 insert to vector). Sequence data also show that the T4 DNA polymerase treatment of the PCR products removed a few bases at the end of the PCR product.

This is an acceptable approach because there are only a couple of bases removed.

The missing base in the AI-1 primer sequence is quite the mystery, as other sequenced clones show the entire AI-1 primer sequence (see Appendix 5). There may be errors in the sequencing data due to secondary structure although the sequence information at the AI-1 primer location is three deep. Another explanation could be hypothesized based on the action of T4 DNA polymerase fidelity. Both of the mentioned hypothesis are without any support and are offered only to provoke thought.

The amino acid sequence is convincing evidence in support of the function of the cloned sequence in pS118. What remains to be determined is the expression patterns of the sequence in pS118. Northern hybridization data is incomplete at this point due to the limitations on obtaining stylar RNA. Primers to amplify the ribonuclease region of the clone have been designed to help in answering the question of the origin of the RNase sequence and further Northern hybridization experiments will help answer some of these unfinished questions.

The work presented here was clearly successful in the attempt to isolate a clone which could code for a ribonuclease.

APPENDICES

APPENDIX A

APPENDIX A

PROTOCOLS FOR SDS-PAGE

The following protocol is commonly known as the Lammeli system and is given for an acrylamide concentration of 10%T, 2.7%C. When SDS-PAGE was followed by RNase activity staining, beta-mercaptoethanol is ommitted in all steps and the resolving gel contains 2 mg/ml yeast RNA. All buffers, glassware, and equipment were Rnase free when Rnase activity staining was to be performed.

Final Concentrations

	Resolving Gel	Stacking Gel	Tank Buffer
Acrylamide conc.	10%T, 2.7%C	4%T, 2.7%C	
Tris HCL	0.375 M	0.125 M	
Tris Glycine			0.02 M tris base 0.192 M glycine
pH	8.8	6.8	8.3
SDS	0.1%	0.1%	0.1%
Amoniumpersulfate	0.1% (w/v)	0.05% (w/v)	
TEMED	0.07% (v/v)	0.05% (v/v)	

Sample Treatment

To prepare the sample for electrophoresis, combine the dry protein with an equal volume of water and 2x treatment buffer (0.125 M tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). If the protein is already in solution, ommit the water. Put the protein sample in boiling water for 90 seconds, then chill on ice until you are ready to use it. This treated sample can be stored frozen for future runs.

Electrophoretic Conditions

Generally, with a 1.5 mm thick gel, 10 to 50 ul of sample is added to each lane. Electrophoresis is done at a constant current of 30 mA for about 3.5 hours. It is important not to let the dye front fun off the bottom of the gel as this will distort the resolution of the proteins on the gel.

APPENDIX A

Coomassie Blue R-250 Staining

The gel is stained with commassie blue (0.125% coomassie blue R-250, 50% methanol, 10% acetic acid) with gentle shaking for 4 hours. It is then destained for 1 hour with destaining solution 1 (50% methanol, 10% acetic acid) and for six hours in destaining solution 2 (7%acetic acid, 5% methanol).

Rnase Activity Staining

1. After electrophoresis (no 2-mercptoethanol in any solutions), wash the gel in 25% isopropanol in 0.01 M tris-HCl pH 7.0 twice for 10 minutes each with gentle shaking. This removes SDS from the gel.

2. Next, wash the gel in 2 uM $ZnCl_2$, 0.01 M tris-HCl pH 7.0 twice for 10 minutes each. This removes the isopropanol from the gel.

3. Incubate the gel in 0.01 M tris-HCl pH 7.0 at 51 C for 30 minutes to 1 hour. This time can vary as a function of the specific activity of the Rnases on the gel that you wish to detect.

4. Stain the gel in 0.2% toluidine blue in 0.01 M tris pH 7.0 for 10 minutes.

5. Destain the gel in 0.01 M tris-HCl for 1 x 10 minutes and 2 x 20 minutes.

6. Zones of Rnase activity will appear as clear bands on a blue background.

APPENDIX B

APPENDIX B

PROTOCOLS FOR AMPLIFICATION OF RECOMBINANT DNA

PCR Amplification of pUC 19 Inserts

Master mix (keep on ice):

Component	Final conc.
Water	
10X Buffer	1x
$MgCl_2$	1 mM
dNTPs	200 mM each
Forward primer	100 nM
Reverse primer	100 nM
Taq polymerase	1.25 U
Total	48.5

1. Add 48.5 ml of master mix to each 500 ml labeled tube.

2. Add 1.5 ml (15 ng) of template DNA (stock conc. = 10 ng/ml) as a droplet to the side of the tube.

3. Spin down all samples and add approximately 50 ml (or 2 drops) of sterile light mineral oil (Sigma).

4. Add a drop of mineral oil to each well in the Perkin Elmer 480 machine and load samples.

Template DNA can come from either a miniprep or, more directly, from the glycerol stock itself using the following procedure: take 1 ml of the glycerol stock and add it to 20 ml water; boil in heat block for 5 min. Use 1 ml of this as template DNA.

Amplification Conditions:
94°/4 min followed by 30 cycles of 94°/1 min, 60°/1 min, 72°/2 min.

APPENDIX B

Primer Sequences
Forward primer: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
Reverse primer: 5'-TCACACAGGAAACAGCTATGAC-3'

(Primers are available from Promega, but were made by the MSU Macromolecular Structure Facility. They will amplify anything within the multiple cloning site, adding approximately 120 bp to the insert.)

PCR Amplification of lambda gt10 inserts.

The protocol is essentially the same for amplification of lambda gt10 inserts with the following modifications:

Primers for amplification of lambda gt10 inserts:

Forward: 5′ CTTTTGAGCAAGTTCAGCCTGGGTAAG 3′
Reverse: 5′ GAGGTGGCTTATGAGTATTTCTTCCAGGGTA 3′

PCR reaction mix:
1.5 mM $MgCl_2$
500 nM each primer

Amplification Conditions:
25 cycles of 94°/1min, 50°/1min, 72°/1min.

In this protocol, template can come from a plaque. Pick the plaque with a toothpick and then transfer the toothpick to 20 microliters of water, swirl the toothpick to dislodge the phage into the water, then use 1-2 microliters of this as the DNA source in the PCR reaction.

APPENDIX C

APPENDIX C

STANDARD HYBRIDIZATION PROTOCOLS AND SOLUTIONS

Pre-hybridization Solution (100 ml)	20 ml
-42 ml sterile diH2O	8.4
-20 ml 25% dextran sulfate (from 500,000 MW dextran)	4.0
-25 ml 20X SSC	5.0
-5 ml 1 M Tris, pH 8	1.0
-2 ml 0.5 M EDTA	0.4
-4 ml 50X Denhardts solution	0.8
-1 ml 20% SDS (MoBio. grade)	0.2
-1 ml 10 mg/ml salmon sperm DNA, sheared and denatured	0.2

50X Denhardts (200 ml)
-2 g bovine serum albumin (fraction V, Sigma)
-2 g Ficol (type 400, Pharmacia)
-2 g PVP (360,000 MW)
-dissolve in this order, one at a time, heating slightly if necessary
-sterile diH2O to 200 ml
-store in aliquots at -20C

Salmon Sperm DNA (sheared/denatured) (or just buy from Boer. Mann.)
-dissolve to 10 mg/ml in water (overnight)
-add 5M NaCl to 0.1 M final
-extract with phenol:chloroform, then chloroform:isoamyl
-shear DNA by passing through a 17 gauge needle 10 times fast
-precipitate by adding 2 volumes ice-cold EtOH
-wash, dry down, redissolve to 10 mg/ml (use spec. or fluor.)
-boil for 10 min. and quick cool
-store at -20 in 1 ml aliquots

APPENDIX C

2X Wash Buffer (1 liter)
-100 ml 20X SSC
-850 ml diH2O
-50 ml 20% SDS

0.2X Wash Buffer (1 liter)
-10 ml SSC
-965 ml diH2O
-25 ml 20% SDS

Procedure:

1. Wet membrane in 2x SSC.

2. Place the membrane in a hybridization tube with the DNA side facing in.

3. Add 10 to 20 mls of pre-hybridization solution and prehybridize for a minimum of 4hrs. at 60 to 65 C.

4. Add 20ng of labeled probe to the hybridization tube and hybridize a minimum of 6hrs at 60 to 65C. Varying the hybridization temperature will affect the stringency of the hybridization. For more stringent conditions use 65 C.

5. Pour out the pre-hybridization solution and probe into the radioactive waste container.

6. Add 25 mls of 2X wash buffer and wash for 30 minutes at 60C.

7. Dump wash buffer into the radioactive waste and repeat the 2X wash.

8. Wash twice for 30 min with 0.2X wash buffer at 60 C.

9. Remove membrane from the hybridization tube and pat dry between two pieces of Whatman filter paper. Wrap the membrane in saran wrap and place in a cassette with film and develop overnight.

APPENDIX D

APPENDIX D

CALCULATIONS OF YIELD FOR cDNA SYNTHESIS REACTIONS

A = total counts per minute (cpm) in first strand reaction.
B = incorporated cpm in first strand reaction.
C = moles of dCTP in the reaction.

Calculation of yield in grams:

Yield [g] = B/A x C x 4(nucleotides) x 330 (average M.W. of a nucleotide).

Calculation of percent yield:

Y = yield as calculated above.
D = amount of input RNA.
% Yield = Y/D x 100.

APPENDIX E

APPENDIX E

LAYOUT FOR DOT BLOTS SHOWN IN FIGURE 4A AND 4B

	1	2	3	4	5	6	7	8	9	10	11	12
A	pS96	98	pS99	pS100	pS101	pS102	pS103	pS104	pS105	pS106	pS107	pS108
B	pS109	pS110	pS111	pS112	pS113	pS114	pS115	pS116	pS117	pS118	pS119	pS120
C	pS122	pS123	pS124	pS125	pS126	pS127	pS128	pS129	pS130	pS131	pS132	pS133
D	pS134	pS135	pS136	pS137	pS138	pS139	pS140	pS141	pS142	pS143	pS144	pS145
E	pS146	pS147	pS148	pS149	pS150	pS151	pS152	pS153	pS154	pS155	pS156	pS157
F	pS158	pS159	pS160	pS161	pS162	pS163	pS164	pS165	pS166	pS167	pS168	pS169
G	pS170	pS40	pS39	pS41	pS42	pS51	pS52	pS53	pS54	pG29	pG47	pG110
H	pG115	pG207	pG293	empty	pBS(-)	tubulin	pUC118	TE	empty	empty	empty	empty

APPENDIX F

APPENDIX F

DNA SEQUENCE AND AMINO ACID TRANSLATIONS FOR pG AND pS CLONES

The suffix f or r after the clone name indicates forward or reverse sequencing primer. The pS clones were short enough to sequence through to the vector on the far side. The vector sequence was identified and removed. There may be about 20 bases of vector sequence in the pS clones on the near side which was not removed because the vector sequence could not be unambiguously identified here. Where primer sequence could be identified, the primer sequence is marked in bold underline (note: not all primer matches are perfect matches).

APPENDIX F

pG115f Cloning vector pBS minus, cloning site EcorV

```
  I  H  G  L  Y  P  E  K  V  C  K  R  S  L  Y  N  Y  K  V  S
 N  S  W  S  I  P  G  E  G  V  *  T  V  T  L  *  L  *  G  I
E  F  M  V  Y  T  R  R  R  C  V  N  G  H  S  I  T  I  R  Y
5'GAATTCATGGTCTATACCCGGAGAAGGTGTGTAAACGGTCACTCTATAACTATAAGGTAT
        10        20        30        40        50        60
3'CTTAAGTACCAGATATGGGCCTCTTCCACACATTTGCCAGTGAGATATTGATATTCCATA
F  E  H  D  I  G  P  S  P  T  Y  V  T  V  R  Y  S  Y  P  I
 I  *  P  R  Y  G  S  F  T  H  L  R  D  S  *  L  *  L  T  D
  N  M  T  *  V  R  L  L  H  T  F  P  *  E  I  V  I  L  Y  *

  I  S  T  S  G  L  V  E  *  L  D  L  D  S  L  *  L  Q  S  L
 N  L  H  E  W  V  S  *  V  T  R  L  R  L  S  L  I  T  I  T
Q  S  P  R  V  G  *  L  S  D  *  T  *  T  L  F  D  Y  N  H
CAATCTCCACGAGTGGGTTAGTTGAGTGACTAGACTTAGACTCTCTTTGATTACAATCAC
       70        80        90       100       110       120
GTTAGAGGTGCTCACCCAATCAACTCACTGATCTGAATCTGAGAGAAACTAATGTTAGTG
L  R  W  S  H  T  L  Q  T  V  L  S  L  S  E  K  I  V  I  V
 I  E  V  L  P  N  T  S  H  S  S  K  S  E  R  Q  N  C  D  S
  D  G  R  T  P  *  N  L  S  *  V  *  V  R  K  S  *  L  *  K

  F  *  K  K  *  S  S  K  N  H  K  R  S  K  V  H  R  R  L  G
 F  L  K  K  V  E  *  Q  K  S  *  E  K  Q  G  P  Q  K  V  G
F  S  K  K  S  R  V  A  K  I  I  R  E  A  R  S  T  E  G  W
TTTTCTAAAAAAAAGTAGAGTAGCAAAAATCATAAGAGAAGCAAGGTCCACAGAAGGTTGG
       130       140       150       160       170       180
AAAAGATTTTTTTCATCTCATCGTTTTTAGTATTCTCTTCGTTCCAGGTGTCTTCCAACC
K  R  F  F  T  S  Y  C  F  D  Y  S  F  C  P  G  C  F  T  P
 K  *  F  F  Y  L  L  L  F  *  L  L  L  L  T  W  L  L  N  P
  E  L  F  L  L  T  A  F  I  M  L  S  A  L  D  V  S  P  Q  S

  V  V  S  P  V  H  Q  V  Y  H  C  G  P  S  Y  S  R  G  C  P
 S  S  K  P  G  S  P  G  L  P  L  R  A  I  I  L  K  R  L  S
E  *  *  A  R  F  T  R  S  T  T  A  G  H  H  T  Q  E  V  V
GAGTAGTAAGCCCGGTTCACCAGGTCTACCACTGCGGGCCATCATACTCAAGAGGTTGTC
       190       200       210       220       230       240
CTCATCATTCGGGCCAAGTGGTCCAGATGGTGACGCCCGGTAGTATGAGTTCTCCAACAG
L  L  L  G  P  E  G  P  R  G  S  R  A  M  M  S  L  L  N  D
 T  T  L  G  T  *  W  T  *  W  Q  P  G  D  Y  E  L  P  Q  G
  Y  Y  A  R  N  V  L  D  V  V  A  P  W  *  V  *  S  T  T  R

  G  S  E  A  *  L  Y  A  Y  Q  C  S  R  R  V  Q  V  A  G  R
 W  L  R  G  V  A  I  C  L  P  M  Q  S  T  C  S  G  G  R  K
L  A  Q  R  R  S  Y  M  L  T  N  A  V  D  V  F  R  W  Q  E
CTGGCTCAGAGGCGTAGCTATATGCTTACCAATGCAGTCGACGTGTTCAGGTGGCAGGAA
       250       260       270       280       290       300
GACCGAGTCTCCGCATCGATATACGAATGGTTACGTCAGCTGCACAAGTCCACCGTCCTT
Q  S  L  P  T  A  I  H  K  G  I  C  D  V  H  E  P  P  L  F
 P  E  S  A  Y  S  Y  A  *  W  H  L  R  R  T  *  T  A  P  L
  A  *  L  R  L  *  I  S  V  L  A  T  S  T  N  L  H  C  S  P
```

APPENDIX F

```
  L  C  *  C  S  G  A
 A  L  L  V  F  R  C
G  S  A  S  V  P  V  H
GGCTCTGCTAGTGTTCCGGTGCAT 3'
       310       320
CCGAGACGATCACAAGGCCACGTA 5'
A  R  S  T  N  R  H  M
 S  Q  *  H  E  P  A
  E  A  L  T  G  T  C
```

APPENDIX F

pG115R Cloning vector pBS minus, cloning site EcorV

```
  R V P C L F H S T K E L Y K R H C P S S
 S S P V L V P L D E R A L Q A A L P F F
L E S R A C S T R R K S F T S G I A L L
5'CTCGAGTCCCGTGCTTGTTCCACTCGACGAAAGAGCTTTACAAGCGGCATTGCCCTTCTT
        10        20        30        40        50        60
3'GAGCTCAGGGCACGAACAAGGTGAGCTGCTTTCTCGAAATGTTCGCCGTAACGGGAAGAA
E L G T S T G S S S L A K C A A N G K K
 R T G H K N W E V F S S * L R C Q G E E
  S D R A Q E V R R F L K V L P M A R R *

  L T R Y C W I G L S P I V Q D S P L L P
 T H A I L L D R A F A H C P R F P T A A
H S R D I A G S G F R P L S K I P H C C
CACTCACGCGATATTGCTGGATCGGGCTTTCGCCCATTGTCCAAGATTCCCCACTGCTGC
        70        80        90       100       110       120
GTGAGTGCGCTATAACGACCTAGCCCGAAAGCGGGTAACAGGTTCTAAGGGGTGACGACG
V * A I N S S R A K A W Q G L N G V A A
 S V R Y Q Q I P S E G M T W S E G S S G
  E R S I A P D P K R G N D L I G W Q Q G

  P R G E S G P S L S P S V A D H P K D P
 P S W G V R A E S Q S Q C G * S S E R P
P L V G S P G R V S V P V W L I I R K T
CCCCTCGTGGGGAGTCCGGGCCGAGTCTCAGTCCCAGTGTGGCTGATCATCCGAAAGACC
       130       140       150       160       170       180
GGGGAGCACCCCTCAGGCCCGGCTCAGAGTCAGGGTCACACCGACTAGTAGGCTTTCTGG
G E H P T R A S D * D W H P Q D D S L G
 G R P S D P G L R L G L T A S * G F S G
  R T P L G P R T E T G T H S I M R F V W

  A K Q S L A W S A F T * P T T * Y Y E A
 S * A V I G L V S L Y L T N Y L I L R G
Q L S S H W L G Q P L P D Q L P N T T R
CAGCTAAGCAGTCATTGGCTTGGTCAGCCTTTACCTGACCAACTACCTAATACTACGAGG
       190       200       210       220       230       240
GTCGATTCGTCAGTAACCGAACCAGTCGGAAATGGACTGGTTGATGGATTATGATGCTCC
L * A T M P K T L R * R V L * R I S R P
 A L C D N A Q D A K V Q G V V * Y * S A
  S L L * Q S P * G K G S W S G L V V L S

  H Q Q R L A S Q D W P T V A V P R Y D R
 S S T A F S F S G L A D C R S S T V R P
L I N S V * L L R I G R L S Q F H G T T
CTCATCAACAGCGTTTAGCTTCTCAGGATTGGCCGACTGTCGCAGTTCCACGGTACGACC
       250       260       270       280       290       300
GAGTAGTTGTCGCAAATCGAAGAGTCCTAACCGGCTGACAGCGTCAAGGTGCCATGCTGG
E D V A N L K E P N A S Q R L E V T R G
 * * C R K A E * S Q G V T A T G R Y S R
  M L L T * S R L I P R S D C N W P V V T
```

APPENDIX F

```
  R  H  C  L  L  S  L  C  G  S  T  V  A  S  L  E  V  L
 S  P  L  S  T  V  T  L  R  Q  Y  C  G  F  S  R  S  A
V  A  I  V  Y  C  H  S  A  A  V  L  W  L  L  S  K  C  *
GTCGCCATTGTCTACTGTCACTCTGCGGCAGTACTGTGGCTTCTCTCGAAGTGCTGA 3'
      310       320       330       340       350
CAGCGGTAACAGATGACAGTGAGACGCCGTCATGACACCGAAGAGAGCTTCACGACT 5'
D  G  N  D  V  T  V  R  R  C  Y  Q  P  K  E  R  L  A  S
 R  W  Q  R  S  D  S  Q  P  L  V  T  A  E  R  S  T  S
  A  M  T  *  Q  *  E  A  A  T  S  H  S  R  E  F  H  Q
```

APPENDIX F

pG29F Cloning vector pBS minus, cloning site EcorV

```
  R V P C L S Y S D D Q D R T A R N N G R
 S S A V F I I L G * P G S Y G T K Q W E
L E C R V Y H T R M T R I V R H E T M G
5'CTCGAGTGCCGTGTTTATCATACTCGGATGACCAGGATCGTACGGCACGAAACAATGGGA
        10        20        30        40        50        60
3'GAGCTCACGGCACAAATAGTATGAGCCTACTGGTCCTAGCATGCCGTGCTTTGTTACCCT
E L A T N I M S P H G P D Y P V F C H S
 R T G H K D Y E S S W S R V A R F L P L
  S H R T * * V R I V L I T R C S V I P S

  T F C L N I G S I * K Q M T V P K * S R
 N F L P Q H R E H L E T N D S S Q M K P
E L S A S T * G A F R N K * Q F P N E A
GAACTTTCTGCCTCAACATAGGGAGCATTTAGAAACAAATGACAGTTCCCAAATGAAGCC
        70        80        90       100       110       120
CTTGAAAGACGGAGTTGTATCCCTCGTAAATCTTTGTTTACTGTCAAGGGTTTACTTCGG
F K R G * C L S C K S V F S L E W I F G
 V K Q R L M P L M * F C I V T G L H L R
  S E A E V Y P A N L F L H C N G F S A S

  I L P F H S R R G S T H R G I V N V H N
 N T P I S F * E R Q Y P S W Y C * C P Q
E Y S H F I L G E A V P I V V L L M S T
GAATACTCCCATTTCATTCTAGGAGAGGCAGTACCCATCGTGGTATTGTTAATGTCCACA
        130       140       150       160       170       180
CTTATGAGGGTAAAGTAAGATCCTCTCCGTCATGGGTAGCACCATAACAATTACAGGTGT
F V G M E N * S L C Y G D H Y Q * H G C
 I S G N * E L L P L V W R P I T L T W L
  Y E W K M R P S A T G M T T N N I D V V

  Q H I Y K E * A P D V M S V L T L S R S
 P T Y L * G I G S * R D V S T D S Q Q I
T N I F I R N R L L T * C Q Y * L S A D
ACCAACATATTTATAAGGAATAGGCTCCTGACGTGATGTCAGTACTGACTCTCAGCAGAT
       190       200       210       220       230       240
TGGTTGTATAAATATTCCTTATCCGAGGACTGCACTACAGTCATGACTGAGAGTCGTCTA
G V Y K Y P I P E Q R S T L V S E * C I
 W C I * L S Y A G S T I D T S V R L L D
  L M N I L F L S R V H H * Y Q S E A S *

  S
 I
H Q
CATCAG 3'

GTAGTC 5'
M L
 D
  *
```

APPENDIX F

pG29R Cloning vector pBS minus, cloning site EcorV

```
      I  H  G  F  C  L  E  T  V  T  L  T  S  T  S  G  S  L  L  C
    N  S  W  I  L  P  R  D  S  Y  L  D  I  H  V  R  E  P  I  V
  E  F  M  D  S  A  *  R  Q  L  P  *  H  P  R  Q  G  A  Y  C
5'GAATTCATGGATTCTGCCTAGAGACAGTTACCTTGACATCCACGTCAGGGAGCCTATTGT
          10        20        30        40        50        60
3'CTTAAGTACCTAAGACGGATCTCTGTCAATGGAACTGTAGGTGCAGTCCCTCGGATAACA
  F  E  H  I  R  G  L  S  L  *  R  S  M  W  T  L  S  G  I  T
   I  *  P  N  Q  R  S  V  T  V  K  V  D  V  D  P  L  R  N  H
    N  M  S  E  A  *  L  C  N  G  Q  C  G  R  *  P  A  *  Q  A

    L  Q  K  Y  C  V  W  L  *  H  N  N  T  T  M  V  L  P  L  L
   P  S  K  I  L  C  L  V  V  T  *  Q  Y  H  D  G  T  A  S  A
  A  F  K  N  I  V  F  G  C  D  I  T  I  P  R  W  Y  C  L  C
  GCCTTCAAAAATATTGTGTTTGGTTGTGACATAACAATACCACGATGGTACTGCCTCTGC
          70        80        90       100       110       120
  CGGAAGTTTTTATAACACAAACCAACACTGTATTGTTATGGTGCTACCATGACGGAGACG
  G  E  F  I  N  H  K  T  T  V  Y  C  Y  W  S  P  V  A  E  A
   R  *  F  Y  Q  T  Q  N  H  C  L  L  V  V  I  T  S  G  R  S
    K  L  F  I  T  N  P  Q  S  M  V  I  G  R  H  Y  Q  R  Q  Q

    K  *  M  S  I  G  F  I  D  F  I  V  S  I  S  Y  V  G  R  V
   E  M  N  E  Y  R  L  H  *  L  H  C  L  N  L  L  C  G  Q  S
  *  N  E  *  V  S  A  S  L  T  S  L  S  Q  S  P  M  W  A  E
  TGAAATGAATGAGTATCGGCTTCATTGACTTCATTGTCTCAATCTCCTATGTGGGCAGAG
        130       140       150       160       170       180
  ACTTTACTTACTCATAGCCGAAGTAACTGAAGTAACAGAGTTAGAGGATACACCCGTCTC
  S  I  F  S  Y  R  S  *  Q  S  *  Q  R  L  R  R  H  P  C  L
   F  H  I  L  I  P  K  M  S  K  M  T  E  I  E  *  T  P  L  T
    F  S  H  T  D  A  E  N  V  E  N  D  *  D  G  I  H  A  S  D

    S  M  S  H  V  R  H
   L  H  V  S  R  P  A
  S  P  C  L  T  S  G  I
  TCTCCATGTCTCACGTCCGGCATC 3'
        190       200
  AGAGGTACAGAGTGCAGGCCGTAG 5'
  R  W  T  E  R  G  A  D
   E  M  D  *  T  R  C
    G  H  R  V  D  P  M
```

APPENDIX F

pG29f Cloning vector pBS minus, cloning site EcorV

```
  R V P C L S Y S D D Q D R T A R N N G R
 S S A V F I I L G * P G S Y G T K Q W E
L E C R V Y H T R M T R I V R H E T M G
5'CTCGAGTGCCGTGTTTATCATACTCGGATGACCAGGATCGTACGGCACGAAACAATGGGA
        10        20        30        40        50        60
3'GAGCTCACGGCACAAATAGTATGAGCCTACTGGTCCTAGCATGCCGTGCTTTGTTACCCT
E L A T N I M S P H G P D Y P V F C H S
 R T G H K D Y E S S W S R V A R F L P L
  S H R T * * V R I V L I T R C S V I P S

  T F C L N I G S I * K Q M T V P K * S R
 N F L P Q H R E H L E T N D S S Q M K P
E L S A S T * G A F R N K * Q F P N E A
GAACTTTCTGCCTCAACATAGGGAGCATTTAGAAACAAATGACAGTTCCCAAATGAAGCC
        70        80        90       100       110       120
CTTGAAAGACGGAGTTGTATCCCTCGTAAATCTTTGTTTACTGTCAAGGGTTTACTTCGG
F K R G * C L S C K S V F S L E W I F G
 V K Q R L M P L M * F C I V T G L H L R
  S E A E V Y P A N L F L H C N G F S A S

  I L P F H S R R G S T H R G I V N V H N
 N T P I S F * E R Q Y P S W Y C * C P Q
E Y S H F I L G E A V P I V V L L M S T
GAATACTCCCATTTCATTCTAGGAGAGGCAGTACCCATCGTGGTATTGTTAATGTCCACA
        130       140       150       160       170       180
CTTATGAGGGTAAAGTAAGATCCTCTCCGTCATGGGTAGCACCATAACAATTACAGGTGT
F V G M E N * S L C Y G D H Y Q * H G C
 I S G N * E L L P L V W R P I T L T W L
  Y E W K M R P S A T G M T T N N I D V V

  Q H I Y K E * A P D V M S V L T L S R S
 P T Y L * G I G S * R D V S T D S Q Q I
T N I F I R N R L L T * C Q Y * L S A D
ACCAACATATTTATAAGGAATAGGCTCCTGACGTGATGTCAGTACTGACTCTCAGCAGAT
        190       200       210       220       230       240
TGGTTGTATAAATATTCCTTATCCGAGGACTGCACTACAGTCATGACTGAGAGTCGTCTA
G V Y K Y P I P E Q R S T L V S E * C I
 W C I * L S Y A G S T I D T S V R L L D
  L M N I L F L S R V H H * Y Q S E A S *

  S
 I
H Q
CATCAG 3'

GTAGTC 5'
M L
 D
  *
```

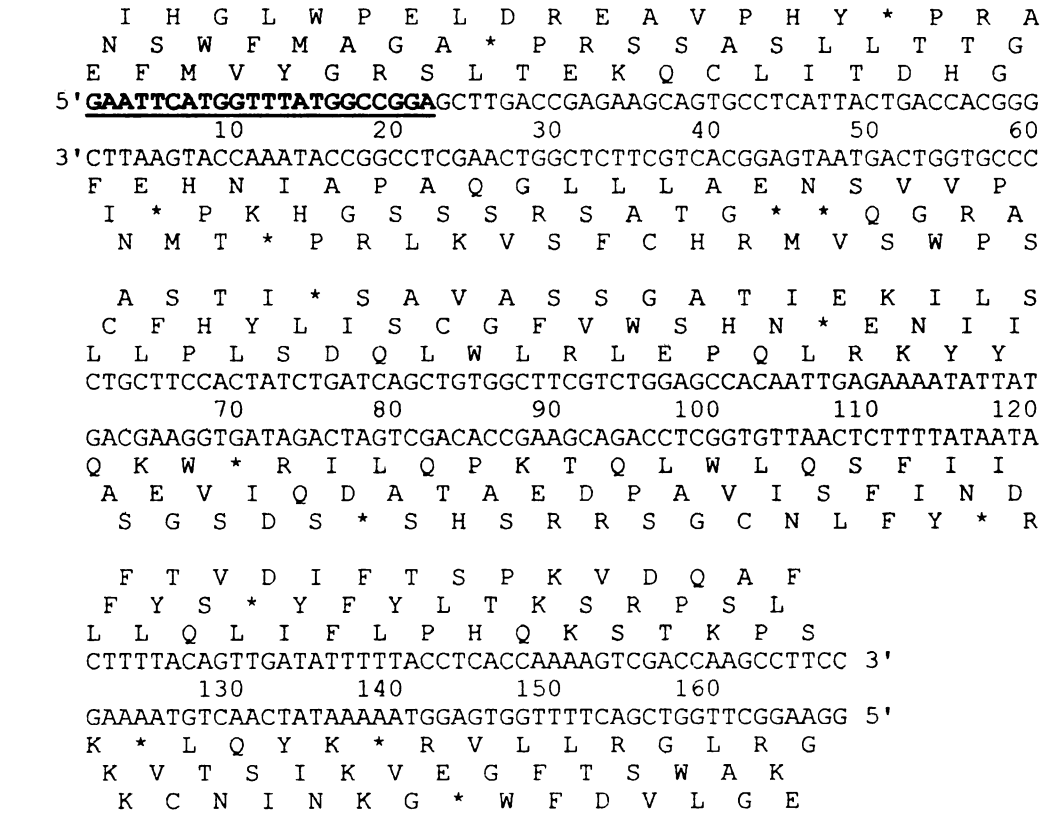
APPENDIX F

pG293f Cloning vector pBS minus, cloning site EcorV

```
   I  H  G  L  C  R  K  I  R  T  R  L  V  Y  L  K  D  I  L  I
  N  S  R  T  V  P  E  D  *  N  P  L  S  L  S  K  R  Y  S  D
 E  F  T  D  C  A  G  R  L  E  P  A  *  F  I  *  K  I  F  *
5'GAATTCACGGACTGTGCCGGAAGATTAGAACCCGCTTAGTTTATCTAAAAGATATTCTGA
         10        20        30        40        50        60
3'CTTAAGTGCCTGACACGGCCTTCTAATCTTGGGCGAATCAAATAGATTTTCTATAAGACT
 F  E  R  V  T  G  S  S  *  F  G  S  L  K  D  L  L  Y  E  S
  I  *  P  S  H  R  F  I  L  V  R  K  T  *  R  F  S  I  R  I
   N  V  S  Q  A  P  L  N  S  G  A  *  N  I  *  F  I  N  Q  Y

   F  W  R  N  T  I  F  E  D  K  Q  T  L  F  L  *  K  E  I  S
  I  L  E  E  Y  H  F  R  G  *  A  D  S  V  F  I  K  R  D  K
 Y  F  G  G  I  P  F  S  R  I  S  R  L  C  F  Y  K  K  R  *
TATTTTGGAGGAATACCATTTTCGAGGATAAGCAGACTCTGTTTTTATAAAAAGAGATAA
         70        80        90       100       110       120
ATAAAACCTCCTTATGGTAAAAGCTCCTATTCGTCTGAGACAAAAATATTTTTCTCTATT
I  K  S  S  Y  W  K  R  P  Y  A  S  E  T  K  I  F  L  S  L
 N  Q  L  F  V  M  K  S  S  L  C  V  R  N  K  Y  F  S  I  L
  K  P  P  I  G  N  E  L  I  L  L  S  Q  K  *  L  F  L  Y  A

   G  K  H  E  T  A  K  S  *  S  H  H  L  Q  D  S  R  P  F  R
  R  E  T  *  N  C  Q  K  L  E  P  P  S  P  R  L  Q  T  F  Q
A  G  N  M  K  L  P  K  A  R  A  T  I  S  K  T  P  D  L  S
GCGGGAAACATGAAACTGCCAAAAGCTAGAGCCACCATCTCCAAGACTCCAGACCTTTCA
       130       140       150       160       170       180
CGCCCTTTGTACTTTGACGGTTTTCGATCTCGGTGGTAGAGGTTCTGAGGTCTGGAAAGT
R  S  V  H  F  Q  W  F  S  S  G  G  D  G  L  S  W  V  K  *
 P  F  C  S  V  A  L  L  *  L  W  W  R  W  S  E  L  G  K  L
  P  F  M  F  S  G  F  A  L  A  V  M  E  L  V  G  S  R  E  S

   A  G  E  V  G  V  C  *  D  G  L  G  S  A  I  V  P  S  P  G
  S  R  *  G  W  S  V  L  R  R  T  R  I  S  Y  R  T  V  A  W
E  Q  V  R  L  E  C  V  K  T  D  S  D  Q  L  S  Y  R  R  L
GAGCAGGTGAGGTTGGAGTGTGTTAAGACGGACTCGGATCAGCTATCGTACCGTCGCCTG
       190       200       210       220       230       240
CTCGTCCACTCCAACCTCACACAATTCTGCCTGAGCCTAGTCGATAGCATGGCAGCGGAC
L  L  H  P  Q  L  T  N  L  R  V  R  I  L  *  R  V  T  A  Q
 A  P  S  T  P  T  H  *  S  P  S  P  D  A  I  T  G  D  G  P
  C  T  L  N  S  H  T  L  V  S  E  S  *  S  D  Y  R  R  R  S

   G  A
  R  G
E  G
GAGGGGCC 3'

CTCCCCGG 5'
L  P
 P  A
  P  G
```

APPENDIX F

pG207R Cloning vector pBS minus, cloning site EcorV



APPENDIX F

pG47f Cloning vector pBS minus, cloning site EcorV

```
  I  H  G  L  W  P  E  L  D  R  E  A  V  P  H  Y  *  P  R  A
   N  S  W  F  M  A  G  A  *  P  R  S  S  A  S  L  L  T  T  G
  E  F  M  V  Y  G  R  S  L  T  E  K  Q  C  L  I  T  D  H  G
5'GAATTCATGGTTTATGGCCGGAGCTTGACCGAGAAGCAGTGCCTCATTACTGACCACGGG
          10        20        30        40        50        60
3'CTTAAGTACCAAATACCGGCCTCGAACTGGCTCTTCGTCACGGAGTAATGACTGGTGCCC
  F  E  H  N  I  A  P  A  Q  G  L  L  L  A  E  N  S  V  V  P
   I  *  P  K  H  G  S  S  S  R  S  A  T  G  *  *  Q  G  R  A
    N  M  T  *  P  R  L  K  V  S  F  C  H  R  M  V  S  W  P  S

  A  F  H  Y  L  I  S  C  G  F  V  W  S  H  N  *  E  N  I  I
 C  F  P  L  S  D  Q  L  W  L  R  L  E  P  Q  L  R  K  Y  Y
L  L  S  T  I  *  S  A  V  A  S  S  G  A  T  I  E  K  I  L
CTGCTTTCCACTATCTGATCAGCTGTGGCTTCGTCTGGAGCCACAATTGAGAAAATATTA
        70        80        90       100       110       120
GACGAAAGGTGATAGACTAGTCGACACCGAAGCAGACCTCGGTGTTAACTCTTTTATAAT
Q  K  G  S  D  S  *  S  H  S  R  R  S  G  C  N  L  F  Y  *
 A  K  W  *  R  I  L  Q  P  K  T  Q  L  W  L  Q  S  F  I  I
  S  E  V  I  Q  D  A  T  A  E  D  P  A  V  I  S  F  I  N  D

  F  Y  M  *  Y  F  Y  L  T  K  S  R  P  S  L  P  Q  I  I  F
 L  L  H  V  I  F  L  P  H  Q  K  S  T  K  P  S  A  D  N  F
S  F  T  C  D  I  F  T  S  P  K  V  D  Q  A  F  R  R  *  F
TCTTTTACATGTGATATTTTTACCTCACCAAAAGTCGACCAAGCCTTCCGCAGATAATTT
        130       140       150       160       170       180
AGAAAATGTACACTATAAAAATGGAGTGGTTTTCAGCTGGTTCGGAAGGCGTCTATTAAA
R  K  C  T  I  N  K  G  *  W  F  D  V  L  G  E  A  S  L  K
 K  *  M  H  Y  K  *  R  V  L  L  R  G  L  R  G  C  I  I  K
  K  V  H  S  I  K  V  E  G  F  T  S  W  A  K  R  L  Y  N  K

  Y  C  P  N  I  R  D  F  *  S  N  N  T  P  H  Q  L  D  P  S
 L  L  P  Q  Y  *  G  F  L  K  Q  *  Y  T  P  P  V  G  P  Q
F  I  A  P  I  L  G  I  F  E  A  I  I  H  P  T  S  W  T  P
TTTATTGCCCCAATATTAGGGATTTTTGAAGCAATAATACACCCCACCAGTTGGACCCCA
        190       200       210       220       230       240
AAATAACGGGGTTATAATCCCTAAAAACTTCGTTATTATGTGGGGTGGTCAACCTGGGGT
K  N  G  W  Y  *  P  N  K  F  C  Y  Y  V  G  G  T  P  G  W
 *  Q  G  L  I  L  S  K  Q  L  L  L  V  G  W  W  N  S  G  L
  I  A  G  I  N  P  I  K  S  A  I  I  C  G  V  L  Q  V  G  T

  I  D  T  S  E  V  K  R  F  L  Q  M  X  D  *  F  V  C  S
 Y  *  Y  *  *  G  Q  E  I  P  P  D  G  G  L  V  R  X  *
V  L  I  L  V  R  S  R  D  S  S  R  W  X  I  S  S  X  V  V
GTATTGATACTAGTGAGGTCAAGAGATTCCTCCAGATGGNGGATTAGTTCGTNTGTAGTA 3'
        250       260       270       280       290       300
CATAACTATGATCACTCCAGTTCTCTAAGGAGGTCTACCNCCTAATCAAGCANACATCAT 5'
Y  Q  Y  *  H  P  *  S  I  G  G  S  P  P  N  T  R  X  Y  Y
 I  S  V  L  S  T  L  L  N  R  W  I  X  S  *  N  T  X  L
  N  I  S  T  L  D  L  S  E  E  L  H  X  I  L  E  X  T  T
```

APPENDIX F

pG293r Cloning vector pBS minus, cloning site EcorV

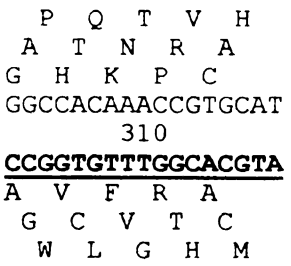
```
     R  V  P  C  L  T  H  S  T  C  F  C  K  S  S  G  V  L  E  M
   S  S  S  V  L  N  T  L  H  L  L  L  Q  K  L  W  S  L  G  D
  L  E  F  R  A  *  H  T  P  P  A  S  A  K  A  L  E  S  W  R
5'CTCGAGTTCCGTGCTTAACACACTCCACCTGCTTCTGCAAAAGCTCTGGAGTCTTGGAGA
         10        20        30        40        50        60
3'GAGCTCAAGGCACGAATTGTGTGAGGTGGACGAAGACGTTTTCGAGACCTCAGAACCTCT
  E  L  E  T  S  L  V  S  W  R  S  R  C  F  S  Q  L  R  P  S
   R  T  G  H  K  V  C  E  V  Q  K  Q  L  L  E  P  T  K  S  I
    S  N  R  A  *  C  V  G  G  A  E  A  F  A  R  S  D  Q  L  H

    A  G  S  Q  L  L  G  Q  F  H  V  S  R  L  S  L  F  L  I  K
   G  W  L  S  A  F  G  T  V  S  C  F  P  L  I  S  L  F  N  K
  W  L  A  L  S  F  W  D  S  F  M  F  P  A  Y  L  S  F  *  *
  TGGCTGGCTCTCAGCTTTTGGGACAGTTTCATGTTTCCCGCTTATCTCTCTTTTTAATAA
         70        80        90       100       110       120
  ACCGACCGAGAGTCGAAAACCCTGTCAAAGTACAAAGGGCGAATAGAGAGAAAAATTATT
  P  Q  S  E  A  K  P  V  T  E  H  K  G  S  I  E  R  K  L  L
   A  P  E  *  S  K  P  C  N  *  T  E  R  K  D  R  K  K  I  F
    S  A  R  L  K  Q  S  L  K  M  N  G  A  *  R  E  K  *  Y  F

    T  E  S  L  I  L  E  M  V  F  L  Q  N  I  R  I  S  F  *  R
   N  R  V  S  Y  P  R  N  G  I  P  P  K  Y  Q  N  I  F  L  K
  K  Q  S  L  L  S  S  K  W  Y  S  S  K  I  S  E  Y  L  S  E
  AAACAGAGTCTCTTATCCTCGAAATGGTATTCCTCCAAAATATCAGAATATCTTTCTGAA
        130       140       150       160       170       180
  TTTGTCTCAGAGAATAGGAGCTTTACCATAAGGAGGTTTTATAGTCTTATAGAAAGACTT
  F  L  T  E  *  G  R  F  P  I  G  G  F  Y  *  F  I  K  R  F
   V  S  D  R  I  R  S  I  T  N  R  W  F  I  L  I  D  K  Q  L
    C  L  R  K  D  E  F  H  Y  E  E  L  I  D  S  Y  R  E  S  S

     *  T  K
    I  N  *  A
   D  K  L  S
   GATAAACTAAGCG 3'
         190
   CTATTTGATTCGC 5'
   I  F  *  A
    Y  V  L  R
     L  S  L
```

APPENDIX F

pS85f Cloning vector pUC118, cloning site HincIII

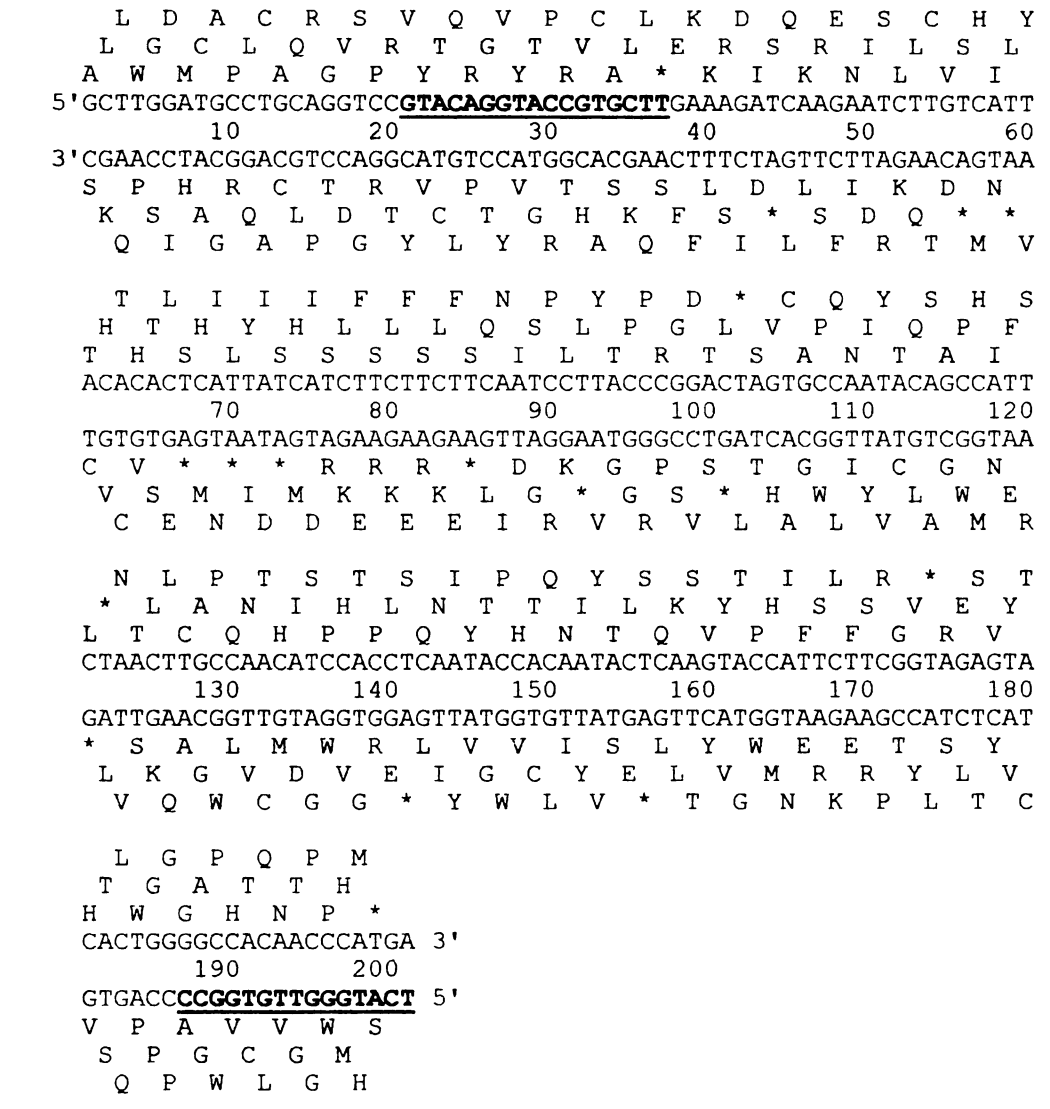
```
   X  Q  V  X  A  X  L  D  P  I  I  T  Q  I  F  F  Q  X  E  A
    S  A  G  X  C  X  X  G  S  H  H  N  T  N  F  F  P  X  G  S
     I  X  R  F  X  P  X  W  I  P  S  *  H  K  F  F  S  K  X  K
5'ATCNGCAGGTTTNTGCCANNTTGGATCCCATCATAACACAAATTTTTTTCCAAANGGAAG
          10        20        30        40        50        60
3'TAGNCGTCCAAANACGGTNNAACCTAGGGTAGTATTGTGTTTAAAAAAAGGTTTNCCTTC
     D  A  P  K  X  W  X  P  D  W  *  L  V  F  K  K  G  F  P  L
      X  C  T  X  A  X  X  S  G  M  M  V  C  I  K  K  W  X  S  A
       X  L  N  X  G  X  Q  I  G  D  Y  C  L  N  K  E  L  X  F  R

        N  W  F  S  A  E  A  A  V  V  E  N  I  *  L  E  V  L  K  G
         E  L  V  *  R  R  G  R  G  R  R  E  Y  L  V  G  G  A  E  G
        R  I  G  L  A  P  R  P  R  S  *  R  I  S  S  W  R  C  *  R
        CGAATTGGTTTAGCGCCGAGGCCGCGGTCGTAGAGAATATCTAGTTGGAGGTGCTGAAGG
                70        80        90       100       110       120
        GCTTAACCAAATCGCGGCTCCGGCGCCAGCATCTCTTATAGATCAACCTCCACGACTTCC
        S  N  T  *  R  R  P  R  P  R  L  S  Y  R  T  P  P  A  S  P
         F  Q  N  L  A  S  A  A  T  T  S  F  I  *  N  S  T  S  F  P
          I  P  K  A  G  L  G  R  D  Y  L  I  D  L  Q  L  H  Q  L  P

           L  M  W  G  S  R  P  M  V  L  T  Q  R  A  G  C  V  X  W  V
            A  D  V  G  X  T  A  D  G  S  N  T  K  S  W  M  R  X  L  G
           G  *  C  G  X  H  G  R  W  F  *  H  K  E  L  D  A  *  X  G
           GGCTGATGTGGGGNTCACGGCCGATGGTTCTAACACAAAGAGCTGGATGCGTGANTTGGG
                  130       140       150       160       170       180
           CCGACTACACCCCNAGTGCCGGCTACCAAGATTGTGTTTCTCGACCTACGCACTNAACCC
           A  S  T  P  X  V  A  S  P  E  L  V  F  L  Q  I  R  S  X  P
            S  I  H  P  X  R  G  I  T  R  V  C  L  A  P  H  T  X  Q  T
             Q  H  P  X  *  P  R  H  N  *  C  L  S  S  S  A  H  X  P  H

              Q  S  G  G  X  S  E  L  Y  A  N  L  H  E  D  I  L  R  *  R
               A  I  R  G  X  L  *  I  I  R  Q  S  P  *  R  Y  S  P  L  K
              C  N  P  G  G  A  L  N  Y  T  P  I  S  M  K  I  F  S  V  E
              TGCAATCCGGGGGGNGCTCTGAATTATACGCCAATCTCCATGAAGATATTCTCCGTTGAA
                     190       200       210       220       230       240
              ACGTTAGGCCCCCCNCGAGACTTAATATGCGGTTAGAGGTACTTCTATAAGAGGCAACTT
              A  I  R  P  X  S  Q  I  I  R  W  D  G  H  L  Y  E  G  N  F
               C  D  P  P  X  E  S  N  Y  A  L  R  W  S  S  I  R  R  Q  L
                L  G  P  P  A  R  F  *  V  G  I  E  M  F  I  N  E  T  S  S


                 I  G  L  A  G  I  L  L  S  Q  V  N  I  Q  S  C  P  L  *  R
                  N  R  T  C  W  D  T  L  I  P  S  K  H  S  I  L  S  F  I  E
                 E  *  D  L  L  G  Y  S  Y  P  K  *  T  F  N  L  V  L  Y  R
                 GAATAGGACTTGCTGGGATACTCTTATCCCAAGTAAACATTCAATCTTGTCCTTTATAGA
                        250       260       270       280       290       300
                 CTTATCCTGAACGACCCTATGAGAATAGGGTTCATTTGTAAGTTAGAACAGGAAATATCT
                 F  L  V  Q  Q  S  V  R  I  G  L  L  C  E  I  K  D  K  I  S
                  I  P  S  A  P  I  S  K  D  W  T  F  M  *  D  Q  G  K  Y  L
                   Y  S  K  S  P  Y  E  *  G  L  Y  V  N  L  R  T  R  *  L  P
```

APPENDIX F


P Q T V H
A T N R A
G H K P C
GGCCACAAACCGTGCAT
310
CCGGTGTTTGGCACGTA
A V F R A
G C V T C
W L G H M

APPENDIX F

pS103f Cloning vector pUC118, cloning site HincIII



APPENDIX F

pS116f Cloning vector pUC118, cloning site HincIII

```
     X  X  P  A  G  P  S  H  G  L  W  P  Q  C  T  L  P  K  N  G
   X  H  X  C  R  S  I  T  W  V  V  A  P  V  Y  S  T  E  E  W
  X  X  X  L  Q  V  H  H  M  G  C  G  P  S  V  L  Y  R  R  M
5'GNTNCATNCCTGCAGGTCCATCACATGGGTTGTGGCCCCAGTGTACTCTACCGAAGAATG
         10        20        30        40        50        60
3'CNANGTANGGACGTCCAGGTAGTGTACCCAACACCGGGGTCACATGAGATGGCTTCTTAC
  X  X  X  Q  L  D  M  V  H  T  T  A  G  T  Y  E  V  S  S  H
   X  M  G  A  P  G  D  C  P  N  H  G  W  H  V  R  G  F  F  P
    X  X  R  C  T  W  *  M  P  Q  P  G  L  T  S  *  R  L  I  T

    T  *  V  L  W  Y  *  G  G  C  W  Q  V  R  M  A  V  L  A  L
   Y  L  S  I  V  V  L  R  W  M  L  A  S  X  N  G  C  I  G  T
  V  L  E  Y  C  G  I  E  V  D  V  G  K  X  E  W  L  Y  W  H
  GTACTTGAGTATTGTGGTATTGAGGTGGATGTTGGCAAGTNAGAATGGCTGTATTGGCAC
         70        80        90       100       110       120
  CATGAACTCATAACACCATAACTCCACCTACAACCGTTCANTCTTACCGACATAACCGTG
  Y  K  L  I  T  T  N  L  H  I  N  A  L  X  F  P  Q  I  P  V
   V  Q  T  N  H  Y  Q  P  P  H  Q  C  T  L  I  A  T  N  A  S
    S  S  Y  Q  P  I  S  T  S  T  P  L  X  S  H  S  Y  Q  C  *

    V  R  V  R  I  E  E  E  D  D  N  X  C  V  M  T  R  F  L  I
   S  P  G  K  D  *  R  R  R  *  *  X  V  C  N  D  K  I  L  D
  *  S  G  *  G  L  K  K  K  M  I  X  S  V  *  *  Q  D  S  *
  TAGTCCGGGTAAGGATTGAAGAAGAAGATGATAATNAGTGTGTAATGACAAGATTCTTGA
        130       140       150       160       170       180
  ATCAGGCCCATTCCTAACTTCTTCTTCTACTATTANTCACACATTACTGTTCTAAGAACT
  L  G  P  L  S  Q  L  L  L  H  Y  X  T  H  L  S  L  I  R  S
   T  R  T  L  I  S  S  S  S  S  L  X  H  T  I  V  L  N  K  I
    D  P  Y  P  N  F  F  F  I  I  I  L  T  Y  H  C  S  E  Q  D


  S
  TCT

  AGA
  R
```

APPENDIX F

pS118 Cloning vector pUC118, cloning site HincIII

```
    C  M  P  A  G  R  T  C  A  V  L  Q  V  D  Y  R  Y  T  *  *
  L  H  A  C  R  S  N  M  C  R  A  S  S  G  L  Q  I  Y  I  I
L  A  C  L  Q  V  E  H  V  P  C  F  K  W  T  T  D  I  H  N
5'CTTGCATGCCTGCAGGTCGAACATGTGCCGTGCTTCAAGTGGACTACAGATATACATAAT
        10        20        30        40        50        60
3'GAACGTACGGACGTCCAGCTTGTACACGGCACGAAGTTCACCTGATGTCTATATGTATTA
K  C  A  Q  L  D  F  M  H  R  A  E  L  P  S  C  I  Y  M  I
 Q  M  G  A  P  R  V  H  A  T  S  *  T  S  *  L  Y  V  Y  Y
  A  H  R  C  T  S  C  T  G  H  K  L  H  V  V  S  I  C  L  L

  M  G  R  *  *  K  L  C  G  P  C  V  L  V  A  C  E  A  L  S
 N  G  E  I  I  K  A  L  R  P  M  R  V  S  G  V  *  S  I  E
K  W  G  D  N  K  S  S  A  A  H  A  C  *  W  R  V  K  H  *
AAATGGGGAGATAATAAAAGCTCTGCGGCCCATGCGTGTTAGTGGCGTGTGAAGCATTGA
        70        80        90       100       110       120
TTTACCCCTCTATTATTTTCGAGACGCCGGGTACGCACAATCACCGCACACTTCGTAACT
F  P  S  I  I  F  A  R  R  G  M  R  T  L  P  T  H  L  M  S
 I  P  L  Y  Y  F  S  Q  P  G  H  T  N  T  A  H  S  A  N  L
  H  P  S  L  L  L  E  A  A  W  A  H  *  H  R  T  F  C  Q  T

  S  C  Q  Y  D  T  S  *  S  I  I  I  L  P  Q  F  L  R  Y  L
 F  L  P  V  *  Y  K  L  E  H  Y  N  T  S  P  I  P  E  V  P
V  L  A  S  M  I  Q  V  R  A  L  *  Y  F  P  N  S  *  G  T
GTTCTTGCCAGTATGATACAAGTTAGAGCATTATAATACTTCCCCAATTCCTGAGGTACC
       130       140       150       160       170       180
CAAGAACGGTCATACTATGTTCAATCTCGTAATATTATGAAGGGGTTAAGGACTCCATGG
N  K  G  T  H  Y  L  N  S  C  *  L  V  E  G  I  G  S  T  G
 E  Q  W  Y  S  V  L  *  L  M  I  I  S  G  W  N  R  L  Y  R
  R  A  L  I  I  C  T  L  A  N  Y  Y  K  G  L  E  Q  P  V  *

  I  T  *  *  A  C  T  D  F  G  R  K  A  R  A  S  *  R  Y  V
 N  N  I  V  G  M  H  G  F  W  P  K  S  *  S  Q  L  K  V  R
*  *  H  S  R  H  A  R  I  L  A  E  K  L  E  P  A  E  G  T
TAATAACATAGTAGGCATGCACGGATTTTGGCCGAAAAGCTAGAGCCAGCTGAAGGTACG
       190       200       210       220       230       240
ATTATTGTATCATCCGTACGTGCCTAAAACCGGCTTTTCGATCTCGGTCGACTTCCATGC
L  L  M  T  P  M  C  P  N  Q  G  F  L  *  L  W  S  F  T  R
 I  V  Y  Y  A  H  V  S  K  P  R  F  A  L  A  L  Q  L  Y  T
  Y  C  L  L  C  A  R  I  K  A  S  F  S  S  G  A  S  P  V  Y  V  K

  T  S  M  H  V  L  H  L  N  D  S  D  R  M  V  G  T  S
 S  Q  N  K  H  A  C  F  T  P  K  *  L  *  Q  N  G  G  H  I
*  S  K  Q  A  C  M  F  Y  T  *  M  T  L  T  E  W  W  A  H
TAGTCAAAACAAGCATGCATGTTTTACACCTAAATGACTCTGACAGAATGGTGGGCACAT
       250       260       270       280       290       300
ATCAGTTTTGTTCGTACGTACAAAATGTGGATTTACTGAGACTGTCTTACCACCCGTGTA
L  *  F  L  C  A  H  K  V  G  L  H  S  Q  C  F  P  P  C  M
 T  L  V  L  M  C  T  K  C  R  F  S  E  S  L  I  T  P  V  D
  D  F  C  A  H  M  N  *  V  *  I  V  R  V  S  H  H  A  C  *
```

APPENDIX F

```
  R  S  T  G  L  H  G  C  G  L  I  I  R  M  A  P  T  H  L  T
 T  K  H  G  T  A  W  L  W  P  N  Y  K  D  G  S  Y  P  S  N
H  E  A  R  D  C  M  V  V  A  *  L  *  G  W  L  L  P  I  *
CACGAAGCACGGGACTGCATGGTTGTGGCCTAATTATAAGGATGGCTCCTACCCATCTAA
       310        320        330        340        350        360
GTGCTTCGTGCCCTGACGTACCAACACCGGATTAATATTCCTACCGAGGATGGGTAGATT
V  F  C  P  V  A  H  N  H  G  L  *  L  S  P  E  *  G  D  L
 R  L  V  P  S  C  P  Q  P  R  I  I  L  I  A  G  V  W  R  V
  S  A  R  S  Q  M  T  T  A  *  N  Y  P  H  S  R  G  M  *  S

  V  I  P  I  V  S  S  T  N  L  R  S  Q  S  *  *  A  T  W  K
 C  D  P  D  S  L  F  D  K  S  E  I  S  E  L  M  S  N  L  E
L  *  S  R  *  S  L  R  Q  I  *  D  L  R  A  N  E  Q  P  G
CTGTGATCCCGATAGTCTCTTCGACAAATCTGAGATCTCAGAGCTAATGAGCAACCTGGA
       370        380        390        400        410        420
GACACTAGGGCTATCAGAGAAGCTGTTTAGACTCTAGAGTCTCGATTACTCGTTGGACCT
Q  S  G  S  L  R  K  S  L  D  S  I  E  S  S  I  L  L  R  S
 T  I  G  I  T  E  E  V  F  R  L  D  *  L  *  H  A  V  Q  F
  H  D  R  Y  D  R  R  C  I  Q  S  R  L  A  L  S  C  G  P  F

  R  T  G  R  H  *  A  A  Q  A  A  M  G  S  G  S  G  P  M  N
 K  N  W  P  S  L  S  C  P  S  S  N  G  F  R  F  W  S  H  E
K  E  L  A  V  T  K  L  P  K  Q  Q  W  V  Q  V  L  V  P  *
AAAGAACTGGCCGTCACTAAGCTGCCCAAGCAGCAATGGGTTCAGGTTCTGGTCCCATGA
       430        440        450        460        470        480
TTTCTTGACCGGCAGTGATTCGACGGGTTCGTCGTTACCCAAGTCCAAGACCAGGGTACT
F  F  Q  G  D  S  L  Q  G  L  L  L  P  N  L  N  Q  D  W  S
 L  V  P  R  *  *  A  A  W  A  A  I  P  E  P  E  P  G  M  F
  S  S  A  T  V  L  S  G  L  C  C  H  T  *  T  R  T  G  H  I

  G  K  S  T  E  H  A
 W  E  K  H  G  T  C
M  G  K  A  R  N  M  L
ATGGGAAAAGCACGGAACATGCTC 3'
       490        500
TACCCTTTTCGTGCCTTGTACGAG 5'
H  S  F  C  P  V  H  E
 P  F  L  V  S  C  A
  P  F  A  R  F  M  S
```

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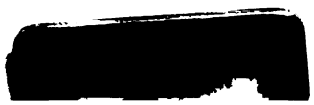

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