The Identification of Recombinant Plants between Molecular Markers Linked to the Ripening-inhibitor (*rin*) and Macrocalyx (*mc*) Genes in Tomato -

Part of a Map-Based Cloning Approach to Locating Genes of Interest

Jennifer L. Umphress

University Undergraduate Research Fellow, 1994-95

Texas A&M University

Department of Biochemistry

APPROVED

Undergraduate Advisor _____

Exec. Dir., Honors Program _____

INTRODUCTION

The tightly linked ripening-inhibitor (*rin*) and macrocalyx (*mc*) loci represent two important developmental genes in tomato fruit ripening and flower development, respectively. Map-based cloning experiments are currently underway to isolate cDNA clones that may correspond to the wild-type homologs of these loci. The first step to confirming possible candidate clones is the complete genetic segregation in every instance with the target genes in a segregating population. For map-based cloning, the only area of interest in the genome is the immediate area of the target genes. Preliminary screening of the segregating population for those individuals with recombinations between genetic markers flanking the target loci can eliminate the need for screening large populations once candidate cDNA clones have been identified. Finding these recombinant plants is one part of a continuing effort by the Giovannoni lab to identify and clone the *rin* and *mc* loci. Once cloned, these genes will serve as tools to both understand and manipulate the ripening process in tomato and additional fruit crop species.

BACKGROUND

The ripening-inhibitor (rin) gene and fruit ripening

The development of fruit by the angiosperms represents a significant step in plant evolution. Unlike the earlier gymnosperms, flowering plants are not limited to chance and wind for the dispersal of seeds. Fleshy fruits are enlarged ovaries that protect the maturing seed. After the seeds are developed, the fruit aids in their dispersal. Fruit

ripening assists in seed dispersal by causing the fruit to soften and accumulate sugars, making the fruit susceptible to attack by microbes or, more frequently, desirable edible food for animals. The use of animals to carry seeds away from their point of origin has helped angiosperms to become the most successful of all plants (Campbell, 1990.)

Fruit development in tomato is an example of climactic ripening. An autocatalytic ethylene burst and a dramatic increase in respiration are associated with the ripening process. The biochemical pathway for the production of ethylene has been identified for most higher plants, and some of the genes responsible have been characterized (ACC Synthase, ACC Oxidase, E8) (Theologis, 1992.) The ripening-inhibitor mutant (*rin*) represents a mutation upstream from currently classified ripening gene in the developmental cascade. Detached fruit from the *rin* mutant never soften, accumulate the characteristic red carotenoid lycopene, synthesize ripening-related ethylene, or ripen in response to exogenous ethylene. Although the fruit is green at seed maturity, it turns yellow with time, and can accumulate 10-20% of normal lycopene levels and a slight increase in softness when exposed to ethylene while still on the vine (Mizrahi et al., 1975 and Buescher, 1977.) The difference in response to exogenous ethylene between fruit on the vine relative to detached fruit indicates that some interactions on the whole-plant level play a role in ripening (reviewed in Giovannoni, 1993.) The *rin* mutant is an interesting target for molecular study because it effects many different aspects of fruit ripening: color, firmness, and interactions with the whole plant.

The macrocalyx (mc) gene and flower development

Our attempts at explaining flower morphogenesis date back to the work of J. W. Goethe in the late 1700s. Goethe detailed the relationship between flower parts, which, in his words, were 'long since known by scientists'. Today, scientists realize that there is much more to learn from flowering plants at the molecular level. The flower is a sophisticated example of plant evolution. Morphologically, a flower is a compressed shoot with four whorls (Campbell, 1990.) Sepals are the protective leaf-like parts at the base of a flower. They arise from the first whorl of the flower. The collection of all the sepals is called the calyx. Petals make up the other non-reproductive parts of the flower, with the reproductive parts being stamens and carpels. The development of each of these has been studied over several decades. Most recently, the electron micrograph has lead to great advancements in the knowledge of these structures, and molecular genetic analysis has contributed to the understanding of the genetic basis of flower development (reviewed in Coen & Meyerowitz, 1991.)

Most of the work in flower developmental regulation has been done with *Arabidopsis thaliana* and *Antirrhium*. Although these plants are only distantly related, they show a great deal of homology on the genetic level of flower development, so it is likely that most dicotyledonous plants are similar in the regulation of their floral development. Genes involved in the formation of the floral meristem and the differentiation of flower parts have already been characterized (reviewed in Coen & Meyerowitz, 1991), but the biochemistry behind flower organ development remains a mystery.

Isolation and study of the macrocalyx gene can broaden our limited understanding in this area. Plants homozygous for the mutant *mc* allele have an enlarged calyx. The calyx exceeds the corolla in length and can seem bladdery in appearance. In addition, the corolla of the *mc* mutant is underdeveloped and rarely spread at flower maturity (Rick & Butler, 1956). The tomato mutant in this study that carries the *rin* gene demonstrates tight linkage between the *rin* and *mc* genes. Within this cross the *rin* and *mc* phenotypes have never been separated, leading to speculation that the mutation within these plants is a single deletion or inversion (J. Giovannoni, personal communication.) However, the two genes are on separate loci, as complementation tests between other *rin* and *mc* mutant strains prove (Fig. 1)(Robinson & Tomes, 1968.) Ultimately the study of the *mc* gene will lead to an advance in our understanding of flower development and regulation on the molecular level.

Tomato as a Model System

The tomato's genetic variability and relative ease of cultivation and regeneration lent it to genetic study long before the advent of molecular biology. There are eight species within the genus *Lycopersicon*. Of these species, the commonly cultivated *L. esculentum* can hybridize with them all (Rick, 1950.) A wide variety of primitive and wild cultivars are also available for study. In addition, genetic study is enhanced by each of the tomato genome's 12 chromosomes being highly distinguishable. Each chromosome has a characteristic arm length and distribution of heterochromatin and euchromatin (Rick & King, 1975.) Tomatoes are easily cultivated. Their growth and reproduction are not

restricted by daylength, and they usually self-pollinate, although large-scale controlled pollination techniques have been worked out to produce hybrid seed. In addition to seed, tomatoes are easily regenerated by cuttings, graftage, and microspore. Cuttings root quickly and dependably. Graftage allows the study and characterization of interactions between mutant and normal plants, and microspore cultivation leads to the generation of haploid plants. Given all of these advantages, considerable work in tomato genetics has been accomplished.

Most importantly, an extensive map of the tomato genome has been made. During the 1960s and 70s, classical breeders used linkage groups and trisomics to establish a map of over 200 tomato genes (Rick, 1975.) As early as 1973 the *rin* and *mc* genes were identified as single recessive mutations linked to the end of the long arm of chromosome 5 (Rick, 1975.) This map has been expanded by the use of molecular techniques to well over 1000 markers with an average density of 1.5 cM between markers (Tanksley et al., 1992.) The existence of such a high-density map is essential to the isolation and characterization of target genes. In addition, tomato is easily transformed by exogenous DNA through *Agrobacterium tumefaciens* transfer (McCormick et al., 1986; Fillatti et al., 1987), a necessary trait for the final proof of map-based cloning (Giovannoni, 1993.)

Map-based cloning - a technique for physically locating and isolating genes of interest

Map-based cloning is a method for isolating a gene for which only the physical map position and phenotype is known. First, a segregating population is made. This can be done, for example, by crossing a mutant strain and a wild-type strain and self-crossing

the first generation. The resulting F2 segregating population is then used to find markers linked to the target gene using a variety of techniques, including restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, and phenotypic analysis. High-resolution mapping of the area is then carried out to determine the order of the DNA markers on the chromosome and the two markers that most closely flank the target gene (Churchill et al., 1993). The next step involves the use a library of high molecular weight genomic DNA such as YACs (yeast artificial chromosomes). This is a collection of high molecular weight DNA from the organism of interest that has been cut and inserted into an engineered minimal yeast chromosome (Burke et al., 1987.) Like any DNA library, theoretically the entire genome of the organism is contained within the YACs that make up the collection. The radio-labeled DNA markers that flank the gene are used as probes on individuals in the YAC library, and those YACs that contain DNA homologous to the markers are identified. A YAC that contains both flanking markers should contain the gene of interest. By radio-labeling this YAC and using it as a probe against a cDNA library, the location of the gene can be more closely pinpointed. cDNAs that hybridize to the YAC clone containing the gene can be used as RFLP probes within the segregating F2 population. A cDNA may contain the target gene if it cosegregates with every individual in the population (Martin et al., 1993.) Such cDNAs will represent candidates for clones of the target gene.

Candidate cDNA clones can be tested and confirmed to contain the target gene by expression and complementation experiments. An RNA blot can be used to look for differences in gene expression between mutant and normal plants with the candidate

cDNA clones as probes. The mutation in the gene may cause it to make defective mRNA or no mRNA at all, which may be indicative of the target gene. Also, cDNAs can be used as RFLP probes to detect differences between the mutant and normal genomes. These differences may represent the genetic lesions resulting in the mutation. Ultimately, the cDNA clone will be confirmed via complementation with the mutant. To do this, cDNA clones will be placed into the plasmids of *Agrobacterium tumefaciens,* and the bacteria will be allowed to infect a plant showing the mutant phenotype (Zambryski et al., 1984; Herrera-Estrella et al., 1985; Fraley et al., 1986; Rogers et al., 1987.) Plant cells that become infected can then take up the DNA of the isolated clones. If one of the candidate clones contain the wild-type homolog to the target gene, then it will be able to confer the normal phenotype to its transformed cells. These cells could then be regenerated to grow plants with normal phenotypes. Such complementation would prove that the cDNA in question contains the normal homolog to the mutant gene.

Numerous experiments are possible once the gene is cloned. The gene could be sequenced and characterized to determine possible function based on homology. Normal and mutant homologs could be found and sequenced, and the wild-type homolog could be studied to ascertain function and expression in the plant at different times in development. Map-based cloning is an effective way to locate a gene of interest so that it can be manipulated and studied on the molecular level.

Previous and current cloning efforts of the rin *and* mc *genes*

A cross between *L. cheesmanii*, which is normal for the *rin* and *mc* loci, and *L. esculentum*, which shows the *rin* and *mc* mutant phenotypes, was used to obtain a segregating (F2) population. High resolution mapping with DNA pools within this population has lead to the identification of five markers - CT93, Yrin2R, CT63A, Trin9L, and TG503 - within 4 cM of the target genes (refer to Fig.2) (Giovannoni et al., 1995; Churchill et al., 1993.) A YAC tomato library was made by Martin et al. in 1992, and the Giovannoni lab is screening its members for linkage with the known markers. To find candidate cDNA clones of the target genes, cDNA libraries of tomato fruit are being screened with YACs that show linkage to one or more of the markers flanking the *rin* and *mc* genes. Before this study, 22 plants with recombinations between the flanking markers TG503 and CT93 had been identified by Dr. Diane Ruezinsky in the Giovannoni lab. To use the segregating population as an ultimate confirmation tool for identifying cDNA clones of the *mc* and *rin* genes, only the immediate area around the genes is of interest. Eliminating plants with no variation in this area from the population saves greenhouse space and time after the cDNA clones have been identified.

MATERIALS AND METHODS

DNA micropreps of tomato seedlings in the segregating population were prepared, digested with EcoRI, and run out on agarose gels with similarly treated parental DNAs. The DNA was transferred to a nylon membrane and hybridized with CT93. The filters

were stripped of radioactivity, and then re-hybridized with TG503. Plants were scored with a '3' if they segregated with the wild-type parent, a '1' if they segregated with the mutant alleles, and a '2' if they were heterozygous, showing both alleles (refer to Fig. 3). After scoring the resultant data, plants that showed recombination between the markers were transplanted and propagated by cuttings. DNA was extracted from these recombinants, and the fruit was scored for the *rin* phenotype. Fine mapping of the recombinant plants was carried out as above with the remaining markers. Refer to Table 1 for a list of restriction enzymes and their respective markers. Progeny of plants producing wild-type fruit were analyzed to determine whether the F2 parent plant is heterozygous or homozygous wild-type at the *rin* locus.

DNA isolation

Approximately 3 weeks post-germination, 5-10 apical meristematic leaves were harvested in 1.5 mL Eppendorf tubes for DNA extraction. The isolation buffer was made up of a freshly prepared 1 : 1 : 0.4 solution of DNA Extraction Buffer (0.35 M Sorbitol, 0.1 M Tris-base, 5 mM EDTA; adjusted to pH 7.5 with HCl; 0.02 M Na Bis-sulfite added just before use), Nuclei Lysis Buffer (0.2 M Tris-base, 0.05 M EDTA, 2 M NaCl, 2% w/v CTAB), and 5% sodium sarkosyl solution, respectively. The samples were homogenized in a small volume of isolation buffer at room temperature using a plastic pedestal mounted in a hand power drill. After incubation for 30-60 minutes at 65°C, an equal volume of chloroform/isoamyl alcohol (v/v 24:1) was added and the solution vigorously shaken. After spinning at 13,000g for 5 minutes the aqueous phase was pipetted into a fresh tube

and an equal volume of isopropanol added to precipitate the DNA. The DNA pellet was washed with 70% ethanol, and dried before being resuspended in about 25 μL of deionized water. Samples were heated at 65°C for a half hour and stored at 4°C overnight to ensure complete resuspension. Samples were stored at -20°C if they were not immediately used.

Restriction digests

Each 50 μL reaction contained 25 μL of microprep DNA, 2 mM spermidine, 10 mM DTT, 1X appropriate restriction enzyme buffer, and 25 units of restriction endonuclease. EcoRI, EcoRV (Promega), HincII, and NsiI reactions were carried out at 37°C, and BclI (NEB) were carried out at 50°C. All reactions were allowed to proceed for at least 6 hours but no more than 20 hours before stop solution (6X Loading buffer made up of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water) was added. Samples were stored at -20°C if they were not used immediately for gel electrophoresis.

Gel electrophoresis

Restricted DNA samples were run on large 0.85% agarose gels in 1X NEB buffer at 24 Volts until the dye front reached 8-9 cm past the wells (about 16 hours). Agarose gels stained with ethidium bromide and visualized under UV light to confirm the presence of DNA and efficiency of its restriction digest.

DNA blotting

Agarose gels were depurinated for 10 minutes in 0.25 N HCl solution, rinsed with distilled water, then incubated twice for 20 minutes each with 0.4 N NaOH. The transfer apparatus was set up as in Figure 4, taking care to use plastic strips around the edges of the sponge to ensure that the liquid could only be drawn up through the agarose gel. After transferring overnight, the apparatus was dismantled and the nylon membrane rinsed in 3X SSC for 20 minutes. The filter was stored at 4°C in plastic wrap until used for hybridizations.

Hybridization

Filters were pre-hybridized for at least 4 hours in hybridization buffer at 65°C. The hybridization buffer consisted of 0.75M NaCl, 0.125M citric acid, 0.6% SDS, 0.05M phosphate buffer (pH 7.4), 0.05% polyvinyl pyrrolidone 40 (PVP-40), 0.05% Ficoll, 0.05% bovine serum albumin (BSA), 0.05M EDTA, 1% dextran sulfate, and 0.1% sheared and denatured salmon testes DNA in water. The labeling reaction consisted of: 10 μL of denatured DNA probe; 11 μL total of dATP, dTTP, dGTP and reaction buffer; 0.6 units of the Klenow fragment of DNA Polymerase I, and 3 μL of 32P-dCTP. The labeling reaction was allowed to proceed for 60-90 minutes at 37°C. Then the probe was purified over a column of Sephadex in 1% SDS, 25 mM EDTA, and finally neutralized with an equal volume of 0.4 N NaOH. Labeled probe was added to the DNA filters and allowed to hybridize for at least 36 hours. The filters were washed with SSC and SDS starting at concentrations of 2X and 0.1%, respectively, and increasing in stringency (decreasing in

salt concentration) for three washes or until the filters registered counts less than 2000 cpm. The filters were then wrapped in plastic wrap and exposed to X-ray film with intensifying screens. Stripping of the filters was accomplished by covering the filter with a 0.1% SDS buffer at 95°C for 3 minutes.

Seed extraction

Seeds were extracted from tomato fruit cut in half with a razor blade and washed in a 50% bleach solution for 10 minutes. The bleach solution was rinsed from the seeds in running water for at least 5 minutes, and any remaining bleach allowed to evaporate off of the seed coats overnight before storage in coin envelopes.

RESULTS AND DISCUSSION

Out of 130 plants surveyed, 9 plants with recombinations between CT93 and TG503 were identified. This represents a recombination frequency of 3.5%, which is comparable to previous findings in the Giovannoni lab (Giovannoni et al., 1995.) Continued study of these plants has been difficult due to a tomato mosaic virus infestation. Of these recombinants, only one - #478 - has produced fruit to date. Its fruit displayed a normal phenotype. Because its recombination must be between the Yrin9L and CT93 markers, and these markers flank the gene, the plant may be heterozygous for the *rin* and *mc* genes. Seedlings of its progeny are ready for testing with the CT93 and TG503 markers to determine whether this is the case. Plants #471 and #375-13 have

recombinations outside of the *rin/mc* region - between markers CT63A and Yrin9L. Fine mapping studies of the remaining recombinant plants are ongoing. Please refer to Table 2 for a listing of all plant scoring and hybridization results.

The addition of these plants to the previous number of recombinants allows for the resolution of additional markers linked to the rin and mc loci. Two such markers are already under study. Locating markers closer to the target genes is a 'step' in chromosome walking, eventually leading to the cloning of the genes. Once isolated, the *rin* and *mc* genes could be sequenced and characterized to determine possible gene function based on homology. Normal and mutant homologs could be found and sequenced. The wild-type homologs of both genes could be studied to ascertain function and expression in the plant at different times in development. Anti-sense expression of the mRNA of the wild-type homologs of these genes could be used to block their expression in normal plants. Ultimately the molecular modes of action and placement in biochemical pathways for these gene products will lead to a greater understanding of the flower development and fruit ripening processes.

SUMMARY

The isolation of the tightly-linked genes for the ripening-inhibitor (*rin*) and macrocalyx (*mc*) phenotypes will be important steps in understanding the molecular basis of fruit and flower development. As part of a map-based approach to cloning these genes, 9 plants with recombinations between molecular markers flanking the genes were

identified out of a segregating population of 130 plants. These plants represent genetic recombination around the *rin* and *mc* loci. Candidate cDNA clones of the genes will eventually be tested for cosegregation with the mutant traits among this sub-population of plants. Cloning the *rin* and *mc* genes will allow the study of their functions at the molecular level, and increase our knowledge of plant developmental regulation.

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FIGURE 1: The inabiliby of *mc* mutants with normal fruit to complement the macrocalyx phenotype in *rin/mc* mutants proves that the *rin* and *mc* genes are located at separate loci (Robinson & Tomes, 1968.)

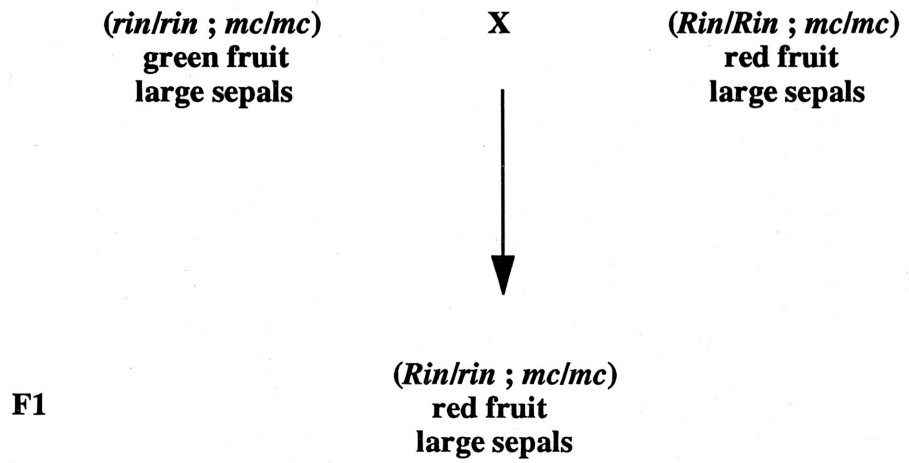

FIGURE 2: Current fine mapping markers to the *rin* and *mc* genes (Giovannoni et al., 1995 and Churchill et al., 1993)

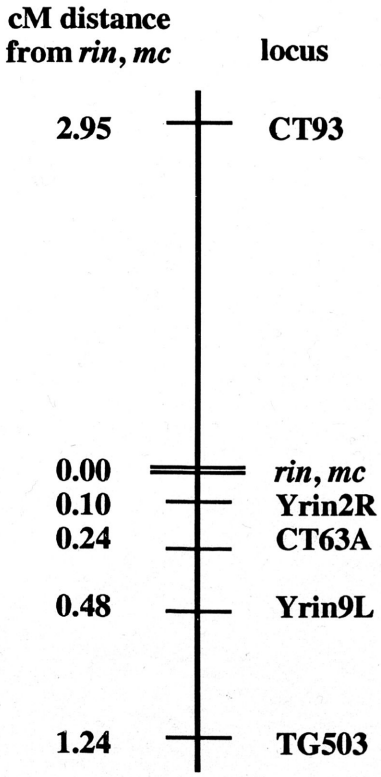

FIGURE 3: A schematic example of actual autorads used in scoring the segregating population for recombination between the markers TG503 and CT93. TG503 and CT93 flank the *rin* and *mc* loci. The same DNA blot was hybridized first with CT93, then stripped and rehybridized with TG503. DNA from each plant was scored according to the alleles it carried. A '1' indicates linkage to the homozygous wild-type phenotype, a '3' the homozygous mutant (*rin* and *mc*) phenotype, and a '2' the heterozygous condition. Lane 1 is DNA from the mutant parent, and lane 2 is from the wild-type parent. The F2 plant in lane 3 is a heterozygote for both the TG503 andCT93 markers. Both markers segregate with the mutant phenotype for the F2 plants in lanes 4, 5, and 7. The plant in lane 6 is homozygous for the mutant phenotype when scored with TG503 but heterozygous when scored with CT93. This plant (#375-13) must have had a recombination event take place between these two markers.

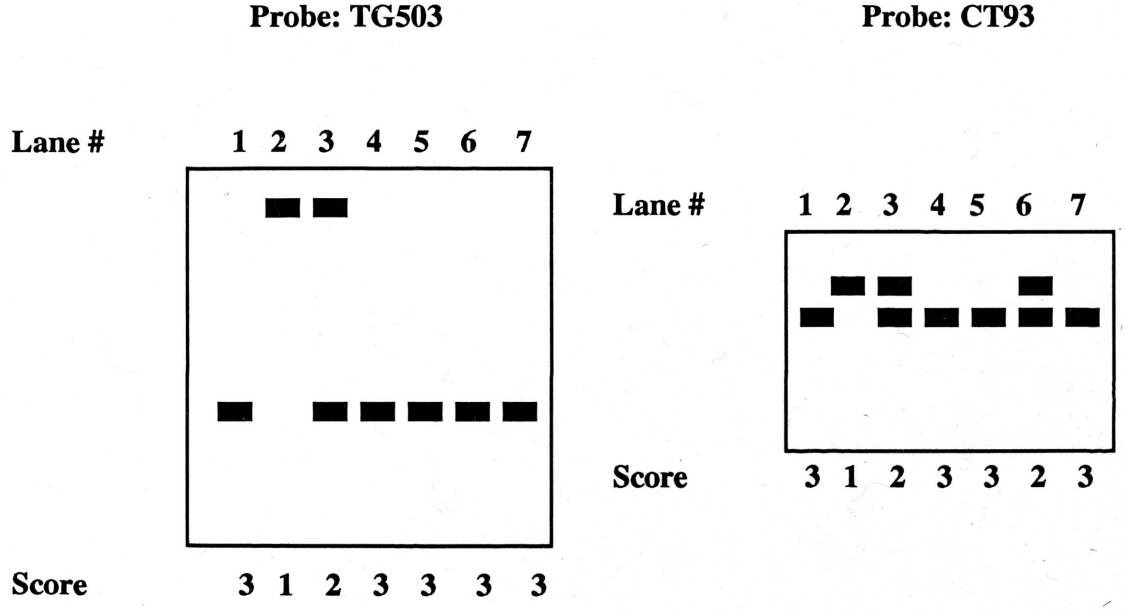

FIGURE 4: DNA blotting apparatus for transfer to a Hybond nylon membrane from an agarose gel

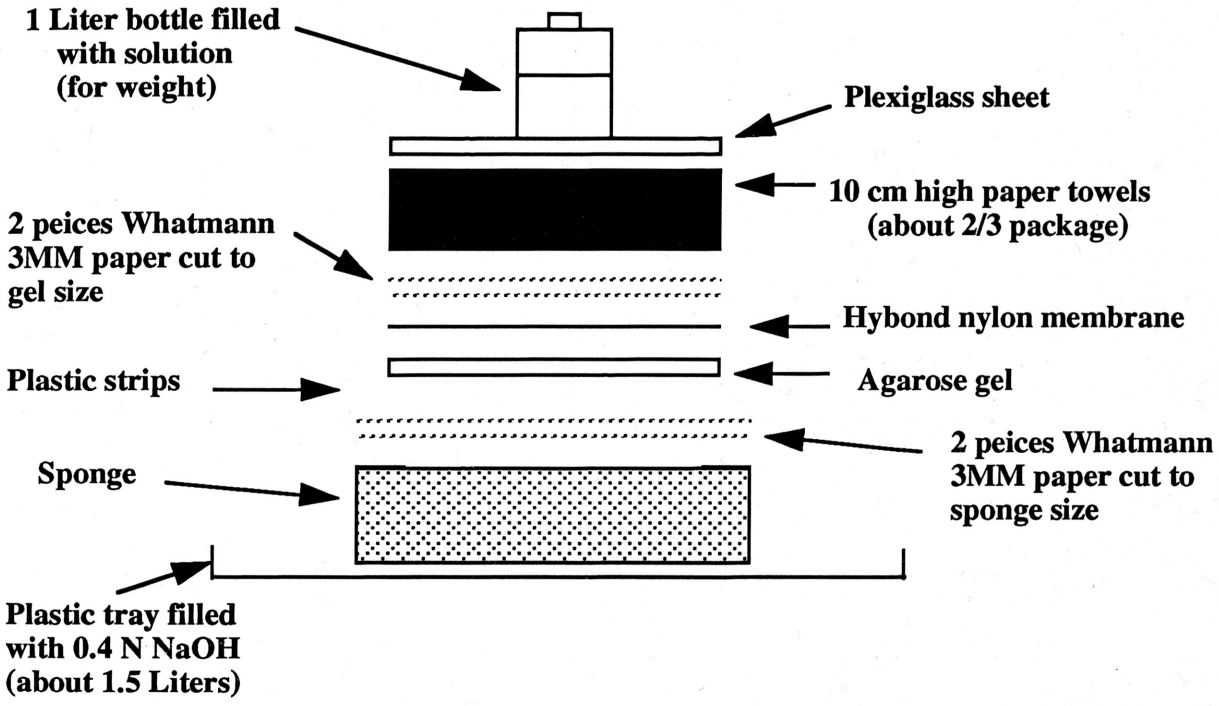

TABLE 1: Restriction enzymes and the molecular markers that show polymorhisms linked to the *rin* and *mc* loci. Refer to Figure 2 for linkage information.

Restriction enzyme	Marker(s)
Eco RI	CT93, TG503
Hinc II	CT63A
Nsi I	Yrin2R
Bcl I	Yrin9L

TABLE 2: Scoring results from the segregating F2 population.

KEY:

'1' = homozygous segregation with the mutant loci
'2' = heterozygous for mutant & wild-type
'3' = homozygous wild-type
'4' = either homozygous wild-type or heterozygous mutant/wild-type

Plant ID	CT93	RIN	Yrin2R	CT63A	Yrin9L	TG503	Comments
427	3					3	
428	2					2	
430	2					2	
431	1					1	
432	2					2	
433	2					2	
434	2					2	
435	2					2	
436	3					3	
437	3					3	
438	1					1	
439	2	2				2	transplanted for seed
440	1					1	
441	2					2	transplanted for seed
442	2					2	transplanted for seed
443	1					1	
444	3					3	
445	2					2	
446	2					2	
447	1					1	
448	3					3	
449	1					1	
450	1					1	
453	2					2	
454	2					2	
455	3					3	
456	3					3	
457	2					2	
458	2					2	
459	2					2	
460	2					2	

Plant ID	CT93	RIN	Yrin2R	CT63A	Yrin9L	TG503	Comments
461	2					2	
462	2					2	
463	2			1	1	1	recombinant
464	2					2	
465	2					2	
466	3					3	
467	2					2	
468	2					2	
469	2					2	
470	3					3	
471	1			1	2	2	recombinant
472	2					2	
473	3					3	
474	2					2	
475	2					2	
476	3					3	
477	3					3	
478	1	4			2	2	recombinant
479	2					2	
480	1					1	
481	3					3	
483	2					2	
484	3					3	
485	3					3	
486	2					3	recombinant
487	2					2	
493	3					3	
495	3					3	
496	3					3	
497	2					2	
498	2					2	
499	2					2	
500	2					2	
501	2					2	
502	1					1	
503	2					2	
504	3					3	
506	2					2	
507	2					2	
508	2					2	

Plant ID	CT93	RIN	Yrin2R	CT63A	Yrin9L	TG503	Comments
509	2					2	
510	1					1	
511	2					2	
512	1					1	
513	2					2	
514	2					2	
515	2					2	
516	1					1	
517	2					2	
518	2					2	
519	2					2	
520	3					3	
521	2					2	
522	2					2	
523	3					3	
524	2					2	
525	2					2	
526	1					1	
527	2					2	
528	2					2	
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531	1					1	
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535	2					2	
536	2					2	
537	2					2	
538	2					2	
539	2					2	
540	1					1	
541	2			2		3	
542	2					2	
543	2					2	
544	1					1	
545	2					2	
546	1					1	
375-1	2					2	
375-3	2					2	

Plant ID	CT93	RIN	Yrin2R	CT63A	Yrin9L	TG503	Comments
375-8	3					3	
375-9	3					3	
375-13	2			2	3	3	recombinant
375-15	3					3	
375-16	2					2	
375-18	2			2	2	3	recombinant
375-19	2					2	
375-20	3					3	
375-22	2					2	
375-23	2					2	
375-25	1			2	2	2	recombinant
375-26	2			1		1	recombinant
375-27	2					2	
375-31	2					2	
375-32	2					2	
375-33	2					2	
375-34	1					1	
375-38	2					2	
375-41	2					2	