Molecular genetic studies in *Fragaria* species: *Agrobacterium*-mediated transformation and fine mapping of the *Phytophthora fragariae* resistance gene *Rpf1*.

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Propositions (Stellingen)

1) The ability of strawberry plants to grow circumpolarly, varying dramatically in ploidy number but still able to intercross, makes strawberries one of the most unique species to work with. this thesis

2) One of the problems of genetic work with strawberries is the ever present argument, whether chromosome pairing is bivalent or multivalent formation. Seneyake and Bringhurst 1967 vs. Mok and Evan 1973. this thesis

3) The commercial strawberry being an autoallopolyploid species is not just genetically complex but the inability to distinguish differences in both chromosome size and staining makes it difficult to combine cytogenetic and molecular genetic mapping results. this thesis

4) Computer programs such as Joinmap and MapMaker, allow for the quick construction of linkage maps but one should always know how to determine linkage among markers by hand, head, pencil, paper and the extremely helpful tables from Allard's paper of 1956. this thesis

5) The fact that the amount of fungicides that the state of California uses for growing strawberries is greater than the combined rest of the worlds usage for treating strawberries and makes a strong case for the development of resistant plants within the United States.

6) A marker-assisted selection program is only as reliable as the disease tests on the mapping population.

7) When doing transformation, cloning or similar experiments, one should remember to consider the possible moral dilemmas of ones own work.

8) Knowledge and experience do not necessarily speak the same language. Taoist philosophy

9) Computers are like people, they are never available when you need them the most.

10) If you cannot say what you mean, then you can never mean what you say. Confucius

11) When governments need to make constitutional amendments to balance a budget the people should question their ability to govern. The people should demand that the government does the work they were elected by the people to do and not create new laws to remind them to do it.

12) Strawberry fields forever. The Beatles, 1967

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General Introduction

The Strawberry

Strawberry plants are herbaceous perennials that reproduce both sexually by seeds, and vegetatively through runners. The true fruits are small seed-containing achenes which are attached to the fleshy red receptacle, the so called berry. The plant crown is a shortened stem, with leaves generally trifoliate, and axially buds borne in a restricted area of the apex. These may give rise to runners, new crowns or inflorescences, depending on the environmental conditions.

Today the commercial strawberry is one of the most important soft fruit crops. The strawberry is mainly grown in the temperate and sub-tropical regions of the world. Twenty-five percent of world strawberry production is concentrated in North America (Hancock and Bringhurst 1988). In the United States, the largest commercial strawberry industry is located in California (Bringhurst and Voth 1989). About 85% of strawberries grown in the USA are from California, about 10% from Florida and the remaining 5% are mainly pick-your-own farms, mainly situated in the NE. In western Europe about 800,000 metric tons of strawberry are produced annually. Spain and Italy account for half of the production and The Netherlands strawberry yield's are about 25,000 metric tons on 1900 hectares (Rosati 1991). In Japan, the strawberry is the most popular fruit with a gross sale value of about $ 1.2 billion US (Oda 1991). Many strawberries are eaten fresh, in addition they are also processed for canning, for jams and conserves, for freezing and for flavouring of drinks and confectionery.

Taxonomy of the strawberry

Strawberry belongs to the genus *Fragaria*, tribe *Potentilleae* of the family *Rosaceae*. The 19 described *Fragaria* species (Table 1) fall into four natural ploidy groups with the following main representatives: the diploid *F. vesca* ($2n = 2x = 14$), tetraploid *F. orientalis* ($2n = 4x = 28$), hexaploid *F. moschata* ($2n = 6x = 42$), and octoploid *F. virginiana* and *F. chiloensis* ($2n = 8x = 56$), with basic number of $x = 7$. The commercially cultivated strawberry (*Fragaria* x *ananassa* Duch.), is an octoploid of hybrid origin.

Fragaria species are restricted to specific areas within the different continents. The wild octoploid species are mainly distributed in North and South America, but one species is endemic to Asia (*F. orientalis*) (Staudt 1989) (Table 1). *F. chiloensis*, the beach strawberry, occurs along the coast of

Chile, ranging from the sea into the Cordillera, South Chile and to a limited extent into Argentina. In North America it ranges from the central coast of California into the Aleutian Peninsula of Alaska. *F. virginiana*, the meadow strawberry, occurs naturally along the Atlantic coast and inland parts of eastern North America (Staudt 1989). *F. ovalis* occurs from New Mexico to Alaska.

The polyploid species of *Fragaria* are in principle all trioecious, plants produce staminate, pistillate, and perfect flowers (Ahmadi and Bringhurst 1991). Unlike dioecious plants in which the male is heterogametic, octoploid *Fragaria* females are heterogametic, segregating 1:1 in progenies and the hermaphrodites are "aberrant males" since they breed true.

Table 1: Summation of *Fragaria*, strawberry species, ploidy level and the origin of development. This table is adapted from Staudt (1989).

Species	Ploidy level	Reproduction	Geographical Distribution
F. daltoniana	2n=2x=14	bisexual	Himalayas
F. iinumae	2n=2x=14	bisexual	Japan
F. mandschurica	2n=2x=14	bisexual	Manchuria and Korea
F. nilgerrensis	2n=2x=14	bisexual	South East Asia
F. nipponica	2n=2x=14	bisexual	Islands of Honshu and Yakushima
F. nubicola	2n=2x=14	bisexual	Himalayas
F. vesca	2n=2x=14	bisexual	circumpolar
F. viridis	2n=2x=14	bisexual	Europe, East central Asia
F. yezoensis	2n=2x=14	bisexual	Japan (Island of Yezo)
F. corymbosa	2n=4x=28	dioecious	North & South China
F. moupinensis	2n=4x=28	bisexual	W. China
F. orientalis	2n=4x=28	bisexual	East Asia
F. moschata	2n=6x=42	dioecious	Europe
F. chiloensis	2n=8x=56	dioecious*	North & South America; Hawaii
F. iturupensis[1]	2n=8x=56	dioecious	Kurile Island
F. ovalis (glauca)	2n=8x=56	dioecious	North America
F. platypetala[1]	2n=8x=56	dioecious	North America
F. virginiana	2n=8x=56	dioecious	North America
F. x *ananassa*[2]	2n=8x=56	dioecious	Hybrid

* some *F. chiloensis* reproduce bisexually
1 = subspecies of *F. virginiana*
2 = hybrid between *F. virginiana* x *F. chiloensis*

History and Breeding of Cultivated Strawberries

In 1768 Duchesne documented the beginnings of the cultivated strawberry (Maas 1984; Staudt 1989; Ahmadi and Bringhurst 1991). Strawberry breeding began in Europe at the beginning of the 18th century when two American species were introduced, *F. virginiana* from North America and female plants of *F. chiloensis* from Concepcion, Chile, introduced by A.F. Frezier in 1712 (Maas 1984). *F. virginiana* and *F. chiloensis* hybridised naturally in various European gardens giving rise to the "Pine Strawberry", later designated as *F.* x *ananassa*. Modern commercial strawberries have since been backcrossed with *F. virginiana* and *F. chiloensis* to introduce other desired traits (Sjulin and Dale 1987; Bringhurst 1990; Ahmadi et al. 1990).

Besides *F. virginiana*, *F. chiloensis* and *F. ovalis*, no other species have been involved in the origin of the modern commercial strawberry cultivars (Bringhurst and Voth 1982, 1984; Scott and Lawrence 1975). The diploid *F. vesca* was by them regarded as poor because it had no attractive properties for commercial production.

Cytogenetics of the strawberry

The diploid *F. vesca* genome is defined as AA, while the octoploid strawberry genome (*F.* x *ananassa*, *F. virginiana*, *F. chiloensis* and *F. ovalis*) was initially designated as AAA'A'BBBB according to Senanayake and Bringhurst (1967). The AA genomes came from the diploid (*F. vesca*) while the A'A' could possibly be from a primitive diploid species. Based on cytological and genetic evidence, a modified version of the genomic constitution of the autoallopolyploid octoploid strawberries as AAA'A'BBB'B' was suggested (Bringhurst 1990). No diploids have been found to contain the B or B' genomes and the origin of the autosyndetic BBBB genome is not known. The results of Bringhurst (1990) are still considered controversial and the genomic constitution of the strawberry is still under investigation.

Interspecific crosses made within a given ploidy level commonly give fertile plants, but crosses between different ploidy levels either fail or result in sterile hybrids, the latter due to unbalanced gametes. Another possiblity is chromosomal or genetic imbalances in the endosperm or between zygote and endosperm (Galletta and Maas 1990).

Yarnell (1931) observed by chromosome pairing studies, that the genome of the diploid strawberry was homologous to one set of the octoploid strawberry. Two of the remaining autosyndetic genomes were pairing, indicating that they were largely homologous. Byrne and Jelenkovic (1976)

however, observed that all chromosomes in nine cultivars and 32 S_1 seedlings of *F.* x *ananassa* were associated as bivalents during meiosis and true multivalent formation was absent. They concluded that the cultivated octoploid strawberry chromosomes have undergone "cytological diploidisation". A further study of pentaploid hybrids between *F.* x *ananassa* and *F. nibicola* (an unrelated diploid species) showed bivalents and frequent multivalent association, indicating a homology between ancestral genomes of the octoploid strawberry. Their studies of chromosome pairing of pentaploid hybrids derived from diploid x octoploid crosses, indicated that two pairs of genomes were involved.

A later chromosome pairing study of four (8x) seedling progenies by Ibrahim et al. (1981), agreed with the findings and interpretations of Byrne and Jelenkovic (1976). Isozyme analysis studies gave indication that the genetics of *F.* x *ananassa* behaves in a diploidic fashion (Arulsekar et al. 1981; Kong and Sjulin 1993).

In contrast, Mok and Evans (1971) studied diakinesis to determine if polysomic inheritance was plausible in nine Eastern North American strawberry cultivars. They observed multivalent formations in each cultivar varying from quadrivalents to octovalents. Their data has suggested that homologies exist between genomes of the cultivated strawberry. They concluded that tetrasomic inheritance could be important and should be considered in the interpretation of genetic data of cultivated strawberries.

Morphological markers in the strawberry

Richardson (1914) studied the inheritance of the runnering trait in *F. vesca* and showed that it was dominant to non-runnering. Further studies with *F. vesca* indicated that the trait appears to be controlled by a single gene with the gene symbol designated as *R* (runnering) and *r* (non-runnering) (Brown and Wareing 1965). They also studied the inheritance of seasonal flowering, which reflects daylength sensitivity, and fruit colour. Both traits were shown to be controlled by a single gene: seasonal flowering (*J*) being dominant to perpetual flowering (*j*) and red fruit colour (*C*) dominant over white (*c*) fruit.

In the octoploid *F* x *ananassa* perpetual versus seasonal fruiting appears to be a quantitative trait (Gutteridge 1959a, 1959b, 1964). Ahmadi et al. (1990) reported, however, that a dominant allele induces daylength-neutrality in the octoploids and furthermore, that daylength-neutrality in the alpine strain of *F. vesca* was controlled by up to three recessive genes in contrast to dominance in *F.* x *ananassa*. Hybridisation of short-daylength with daylength-neutral strains of the European *F. vesca* supported the postulation of single gene control of the trait within the European subspecies; however,

their results of crossing the European Alpine strain to the native California *F. vesca* were different and showed involvement of more than two genes (Ahmadi et al. 1990).

Biochemical and molecular markers.

Marker-facilitated selection is a viable method in numerous crop plants for the improvement of disease and insect pest resistance. Various techniques have been employed to map these agronomically important genes, such as isozyme, RFLP and RAPD markers and genotypes which differ only for the gene of interest, e.g. near-isogenic lines. Molecular markers are normally readily reproducible by the polymerase chain reaction (PCR: Sakai et al. 1988). One of the major methods of detecting markers linked to useful genes is by Bulked Segregant Analysis (BSA) described by Michelmore et al. (1991). The usage of RAPD markers in combination with BSA has been used to tag various disease resistance genes of stripe rust, stem rust and leaf rust in barley (Barua et al. 1993; Chen et al. 1994; Borokova et al. 1995; Poulsen et al. 1995), anthracnose and rust resistance in common bean (Haley et al. 1993; Miklas et al. 1993; Adam-Blondon et al. 1994), white rust (*Albugo candida*) in *Brassica napus* (Ferreira et al. 1995), black leaf spot in Chinese elm (Benet et al. 1995), powdery mildew in lettuce (Paran and Michelmore 1993; Paran et al. 1993), potato leaf roll virus and potato cyst nematode in potato (Barker et al. 1994; Pineda et al. 1993), gall midge resistance in rice (Nair et al. 1995), *Pseudomonas* resistance, powdery mildew resistance in tomato (Martin et al. 1991; Michelmore et al. 1991; Chunwongse et al. 1994; Van der Beek et al. 1994), and leaf rust in wheat (Schachermayr et al. 1994).

Isozyme Analysis. Isozymes reflect variability of the DNA sequence through differences in amino acid sequences, that produce a change in electrophoretic mobility. Isozyme polymorphisms provide a useful tool for the evaluation of genetic differences among genotypes and for linkage mapping. The alleles for most isozyme markers interact in codominant fashion, heterozygotes being distinguishable from either homozygote. Isozymes rarely exhibit epistatic interactions or pleiotropic effects (Tanksley and Rick 1980). However, the value of isozymes as genetic markers is limited by the absence of sufficient numbers to saturate a genetic map (Helentjaris et al. 1986). Arulsekar and Bringhurst (1981, 1983) and Williamson et al. (1995) studied isozyme genetics in the diploid species of *Fragaria* as a model to understand isozyme inheritance in the polyploids. Yu and Davis (1995) and Williamson

et al. (1995) studies on the diploid, *F. vesca* report linkage of SKDH isozyme to red vs. yellow fruit colour and PGI to runnering vs. non-runnering.

Restriction Fragment Length Polymorphism (RFLP). Restriction enzymes recognise specific short sequences of (usually) unmethylated DNA and cleave the duplex. Botstein et al. (1980) proposed randomly-dispersed restriction fragment length polymorphisms (RFLPs) as a new source of genetic markers in humans. Genomic DNA from two genetically distinct individuals is digested with a restriction enzyme, separted by gel electrophoresis, blotted onto a membrane, and probed with labelled DNA clone; polymorphisms in the hybridisation patterns are the results of DNA sequence differences between the individuals. The analysis utilises these DNA sequence variations as alleles of which the inheritance can be followed in the same manner as conventional markers. RFLP markers are generally codominant, locus specific, not subject to pleiotropic effects and have developmental stability (Havey and Muehlbauer 1989). Unlike isozymes, RFLPs are potentially unlimited in number, allowing a much wider use of the molecular marker approach.

RFLPs have been used to construct saturated linkage maps where the density of markers is so high that any gene of interest is likely to cosegregate with flanking DNA markers (Williams et al. 1991). RFLP maps have been developed for a number of species like maize (Helentjaris et al. 1985), tomato and potato (Bonierbale et al. 1988; Tanksley et al. 1992) and peanut (Halward et al. 1992).

Random Amplified Polymorphic DNA (RAPD): Williams et al. (1990) and Welsh and McClelland (1990) demonstrated that short 10 bp length single primers of arbitrary nucleotide sequence could be used to amplify segments of genomic DNA from a variety of species using PCR technology. Polymorphisms among the amplification products are typically detected as DNA segments which amplify from one parent but not the other and can be used to construct genetic maps.

Random amplified polymorphic DNA (RAPD) markers can be used for genetic mapping, for DNA fingerprinting, population genetics, and identification of chromosome-specific DNA fragments. The dominant nature of RAPD markers does not allow the complete determination of genotypes in the segregating population and would lead to an inaccurate map when the female and male parent maps are combined together. Only rarely are codominant RAPD markers detected (Williams et al. 1990). Although the genetic information content of a single, dominant RAPD marker is lower than for codominant isozyme and RFLPs, when many of these markers are used to

define a genome they begin to have enormous utility because the are easy to detect, inexpensive to test and can quickly be used to saturate a specific region of the genome. RAPD markers have been used to map a wide variety of plant genomes (RAPD: for reviews see Devos and Gale 1992; Waugh and Powell 1992; Tingey and Del Tufo 1993) and for gene tagging or map-based cloning of plant disease-resistance genes (Jones et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994). RAPD-PCR has been used to a limited extent in the commercial strawberry for cultivar and genotype identification (Gidoni et al. 1994; Levi et al. 1994; Parent and Pagé 1994) and in the diploid strawberry for mapping (Haymes 1993; Davis and Haymes 1993; Davis and Yu 1997).

Other types of codominant markers are sequence tagged sites (STS) (Olsen et al. 1989), and sequence tagged microsatellite site (STMS) markers from microsatellites which amplify oligonucleotide repeats of variable length (Beckman and Soller 1990). A STS is a short, non-repetitive unique single-copy DNA sequence amplified by PCR from a genomic library or genomic DNA using specific oligonucleotide primers that identifies a known location on a chromosome. Physical mapping with STS may be done using PCR or by hybridisation, since they do not contain repetitive DNA by their original definition.

The technique of Konieczny and Ausubel (1993) uses co-dominant, cleaved amplified polymorphic sequences (CAPS) to determine the genotype of origin of the PCR products. Usually the PCR products for the STS markers are cleaved by restriction enzymes and then separated by gel electrophoresis; these are then called CAPS.

Paran and Michelmore (1993) describe a type of marker very similar to the STS called, sequence characterised amplified regions (SCAR) markers. They differ from STS in the respect that they are defined genetically; therefore they can be used as physical landmarks in the genome and as genetic markers. A SCAR can contain repetitive DNA sequences within the amplified fragment as they are assessed by PCR only and then run directly on a gel after PCR amplification. Each of these markers (STS, CAPS, and SCARs) are powerful tools and can quickly be integrated into a breeding programme.

AFLP (amplified fragment length polymorphism) represent a new type of marker, developed as a DNA fingerprinting technique by Zabeau and Vos (1993) and Vos et al. (1995) that is fast, highly reproducible and produces in a short time large amounts of data. AFLP markers, like RAPDs, are in most cases dominant markers and can be converted into co-dominant markers by cloning, sequencing and primer design. AFLP markers are genomic restriction fragments detected after selective amplification using PCR. Based on the recognition sequence of the restriction enzymes used and on arbitrary chosen bases at the 3´ end of both primers, sets of 100-200

restriction fragments are selectively amplified and 100-200 AFLP loci can be detected in a single experiment.

Resistance Breeding Methods in Strawberry

Various breeding methods have been utilised to obtain desired characteristics in the strawberry. Strawberries can be self fertilised, therefore the pedigree method has been widely used, as has the bulk and the backcross method (Briggs and Knowles 1967). A pedigree provides a record of the plant lines of descent of all individuals or lines in each generation. The information provided by this selection such as resistance to disease or pests, vigour, or other useful information is important when decisions of which lines to eliminate must be made (Briggs and Knowles 1967). The pedigree method provides a means of studying the inheritance of some important characteristics, thereby adding further knowledge of the genetics of the crop.

Where a disease or insect is a constant feature of the environment, the bulk method, including composite crosses, allows the use of very large populations (Briggs and Knowles 1967). The backcross method usually requires less time than the other methods. This is important when diseases develop rapidly. More importantly, unlike the other methods, is the possibility in this method of adding many genes for resistance to a variety, without losing the previously added genes. This is only possible if the added traits are at a different locus than those already present.

Another method is the utilisation of molecular markers linked to the gene(s) of interest, allowing indirect selection. An advantage of this approach is the ability to pyramid desired characteristics but only if they are not clustered. This technique can be done at a very early stage in the plants life cycle, as is discussed in Chapter 7.

General background on the fungal disease *Phytophthora fragariae*.

Red stele root rot was first known as Lancashire Disease, for the district of Lancashire in Scotland where it was first observed and studied in 1920. The original source of the infection of the disease is unknown but Moore et al. (1964) found that five of nineteen species of *Potentilla* were susceptible to red stele when inoculated artificially. They speculate that *Potentilla* might serve as the natural host of the disease.

The commercial strawberry (*Fragaria* x *ananassa*) is susceptible to red stele root rot caused by the fungus *Phytophthora fragariae* var. *fragariae*. *P. fragariae* belongs to the division *Oomycota* and occurs in regions with wet winters and soils with poor drainage (Paulus 1990).

Oospores allow the fungus to survive in soil for long a period after which they produce sporangia and zoospores. Secondary metabolites exuded from the root tips attract the zoospores of *P. fragariae* which then infest the plant. The zoospores encyst in large numbers on the roots, produce germ tubes and penetrate the epidermis. Within three days the fungus reaches the differentiating phloem and pericycle. Symptoms of the disease are the stele becomes red in colour, infection of the roots, dwarfism, wilting and eventually plant death.

In North America and Europe, red stele is controlled by the use of clean planting stock, cultural practices that reduce the risk of an outbreak of the disease, fungicides, and resistant cultivars. Montgomerie (1984) noted that *P. fragariae* is introduced into new areas by infected plant stock. Strawberry nurseries that use micropropagation in the plant production have decreased the risk of *P. fragariae* infecting the material (Nickerson and Maas 1991). Chemical control of red stele with methylbromide and chloropicrin reduce the inoculum potential of the fungus in the soil but does not eliminate it completely (Jeffers 1957). Metalaxyl (Ridomil; Ciba-Geigy) another fungicide which is and has been used in North America (mainly Canada) and in north-western Europe has been effective but resistant strains of *P. fragariae* have been reported.

In a 1995 study by the Environmental Working Group, a research and advisory group in Washington D.C. analyzed 15,000 samples of food for pesticides by the Food and Drug Administration during 1992 and 1993. The strawberry ranked number 1, among the 42 fruits and vegetables tested, to seven different measures of what it called pesticide contamination, including the percent of the crop with detectable residues and the potency of the average amount of cancer-causing pesticides found each year on that crop.With the growing concern and pressure from environmental groups towards chemical treatments the use of naturally resistant cultivars will play a more substantial role in future cultivation.

The commercial strawberry as a model for the gene-for-gene interaction theory.

When breeding for disease resistance, two factors should be considered, the host and the pathogen (pest). The genetics of both systems must be kept in mind and the environment with its effect on both the crop and the disease. The gene-for-gene interactions specify plant disease resistance. Resistance is only expressed when a plant containing a specific *R* gene needs a pathogen with the corresponding avirulence gene. All other combinations lead to non-recognition by the host, and the result is disease. The genetic

basis of this hypersensitive response (HR) was first clarified by Flor (1956, 1971) who demonstrated that the resistance of flax to the fungal pathogen *Melampsora lini* was a consequence of the interaction of paired genes in the host and pathogen. His work was the foundation for the gene-for-gene hypothesis of plant-pathogen interactions, mapping of *R* and avirulence (*avr*) genes and for the molecular cloning of plant *R* genes and their corresponding pathogen *avr* genes.

Van de Weg et al. (1989, 1993, 1997) formulated that red stele resistance in strawberries and virulence of *P. fragariae* behaves according to the gene-for-gene system. Five resistance genes and their corresponding avirulence genes have been identified by testing cultivars of strawberry against races of the fungus (Van de Weg 1989, 1993).

Various breeding programmes such as the USDA-ARS, Beltsville, MD (USA), Agricultural Canada (Canada), the Scottish Crop Research Institute (England), and CPRO-DLO (The Netherlands); have been successful in breeding genotypes with resistance to multiple races of *P. fragariae*. The CPRO-DLO and the USDA-ARS breeding programme is based mainly on only three specific combinations of two to three resistance genes (*Rpf1*, *Rpf2* and *Rpf3*). However, classical screening methods are laborious, expensive and sometimes cannot assess the many effective combinations. This latter is sometimes due to epistatic interference where one of the resistance genes obscures the presence of another resistance gene. Therefore, selections with only one such gene are difficult to distinguish from selections in which several resistance genes are combined. It is possible by progeny analysis but this is very time consuming.

Classical breeding methods have already been able to incorporate resistances to various diseases in the commercial strawberry (Maas et al. 1989). However, the ability to pyramid resistance genes and accurately screen them is still problematic. In the commercial strawberry both aspects can be improved by the use of linked molecular markers. Utilisation of molecular markers may allow the ability to pyramid and screen for resistance genes more efficiently than present conventional methods.

Transformation of commercial strawberry

Genetic engineering provides a means by which genes of agronomic interest can be incorporated into the genome of plants while conserving their existing genetic background. Foreign genes can be introduced into plants by various methods such as electroporation, particle bombardment, microinjection, and, *Agrobacterium*-mediated transformation.

James et al. (1990) produced viable transgenic octoploid strawberry (*F.* x *ananassa*) plants using an disarmed *Agrobacterium* binary vector system. The transfer and integration of the nopaline synthase (NOS) and the neomycin phosphotransferase (NPT-II) gene was confirmed by Southern blot analysis and NPT-II gene expression of tissues on kanamycin containing media. They found that petiole tissue was transformed more efficiently than leaf disks. Non-transformed petiole sections regenerated shoots less efficiently than leaf disks, but perhaps in transformation the total number of cells exposed or competent for transformation per explant was greater in petioles than in leaf disks.

Nehra et al. (1990a) used *A. tumefaciens* carrying the binary plasmid pBI121 to produce transgenic *F* x *ananassa* in a callus culture system. Shoots produced from the putative transformed calli were assayed to show that both NPT-II and GUS had been incorporated and were being expressed. Nehra et al. (1990b) developed a protocol to transform *F.* x *ananassa* cv. Redcoat by *A. tumefaciens* carrying plasmid pBI121 using a leaf disk regeneration system. Successful transformation of the plants was shown by the expression of NPT-II and GUS genes in all transgenic clones. Molecular analysis confirmed that both genes were integrated into the strawberry genomic DNA.

Nyman and Wallin (1992) introduced foreign genes into *F.* x *ananassa* protoplasts by electroporation. Viable strawberry protoplasts were isolated and cells were electroporated. Transformed cells were selected by growth in selection medium containing hygromycin B, and GUS activity from callus was assayed by histochemical and Southern blot analysis. Uratsu et al. (1991) studied the susceptibility of the diploid strawberry *F. vesca* to infection by *Agrobacterium* and found that it was possible to use the diploid strawberry for *Agrobacterium*-mediated transformation studies. However, in both studies plants were not regenerated. Mansouri et al. (1996) developed a method for direct shoot regeneration of transformed diploid *F. vesca* but did not examine the stability of the transferred genes by progeny analysis. The diploid strawberry, for studies related to the octoploid species, represents an ideal model system to assess gene transformation.

Outline of this thesis

The aim of this thesis was to develop a genetic map (using the diploid strawberry as a model) with the emphasis towards the development of a high density molecular map of a resistance gene in the octoploid strawberry, and finally how to integrate the genes back into the strawberry germplasm.

A RAPD map was first developed for *F. vesca* and then a map around the *Phytophthora fragariae* resistance gene *Rpf1* of the commercial

strawberry. Specific primers for closely linked marker were developed for selection of *P. fragariae* resistant plants in a breeding programme. The RAPD and the SCARs conservation to the *Rpf1* gene was assessed in European and North American genotypes. To be able to transform strawberry with important agronomical genes, a transformation procedure was developed.

In Chapter 1, the construction of a RAPD and isozyme map of the diploid strawberry *F. vesca*, is presented. The linear order is described for the heterozygous male parent. The diploid strawberry was utilised as the model system for preliminary research before working with the more genetically complex octoploid strawberry.

In Chapter 2, the development of a RAPD map in the commercial strawberry for the region of the *Phytophthora fragariae* resistance gene (*Rpf1*) is reported. This gene, confers resistance to various isolates of red stele root rot. To do this Bulked Segregant Analysis was utilised to screen a segregating population of plants to red stele root rot disease. The problems of the autoallopolyploidic nature of the strawberry was not problematical for mapping due to a disomic segregation of the *Rpf1* region.

In Chapter 3, the construction of specific dominant SCAR markers to the *Rpf1* resistance gene is described. The SCAR markers were designed on sequence homology differences found from utilising a codominant SCAR marker.

In Chapter 4, a simple and efficient DNA isolation protocol for usage in a plant breeding programme is described.

In Chapter 5, The RAPD and SCAR primers for *Rpf1* resistance were screened for their conservation in European and North American strawberry genotypes. The markers were shown to be conserved in resistant genotypes and were useful by indicating when the resistance gene has introgressed or was lost in the strawberry genotypes.

In Chapter 6, utilising the diploid strawberry as a model system for genetic transformation of the commercial octoploid strawberry is described. A stabile system of introducing genes into plants but more importantly to have them passed into the progeny in Mendelian fashion is reported.

Finally, a general discussion connecting how mapping of the diploid strawberry led to fine mapping the *Rpf1* resistance gene in the octoploid strawberry. Various mapping applications are described and how marker-assisted selection can be applied in strawberry breeding programmes.

Account: Chapters 2-6, have either been published or are submitted for publication. Chapter 1 is being updated since additional data are now available and will be submitted at a latter date.

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Chapter 1

Development of a molecular map in *Fragaria vesca* L., the diploid strawberry

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Abstract

The diploid (2n = 2x = 14) strawberry, *Fragaria vesca,* is closely related to the commercial octoploid (2n = 8x = 56) strawberry, *Fragaria* x *ananassa.* Genetic analysis of the commercially cultivated strawberry is difficult due to its complex octoploid genome. Based upon the earlier studies of similarities between the strawberry species, RAPD technique was used to create a partial map of the diploid strawberry. A partial molecular map was constructed on the basis of a segregating F_1 population of 55 strawberry plants using RAPDs and two isozyme (PGI and SKDH) markers. The population was derived from a cross between an inbred Alpine cultivar 'Yellow Wonder' and a highly heterozygous, wild accession FRA 364. Five distinct linkage groups of 6 to 17 markers, and 8 unlinked markers, were identified in the population tested. The diploid strawberry was utilised in a preliminary study as a model system for genetic analysis due to its small genome size and less complex genetic constitution before working with the commercial octoploid species

Key-words: linkage map, isozyme-analysis, PCR, RAPD

Introduction

Genetic analysis of the commercially cultivated strawberry (*Fragaria* x *ananassa* Duchesne) is difficult due to its complex octoploid (2n = 8x = 56) genome (Arulsekar et al. 1981). Various morphological traits have been studied in the diploid strawberry such as flowering habit, fruit colour, and runnering and were shown to segregate independently (Richardson 1914; Brown and Wareing 1965). The only report of genetic linkage in the commercial strawberry is of the *Phytophthora fragariae* (*Rpf1*) resistance gene locus (Haymes et al. 1997). However, in the diploid strawberry linkage was shown between PGI-2 allozyme to runnering and of SKDH to a locus governing red versus yellow fruit colour (Yu and Davis 1995; Williamson et al. 1995) and a RAPD map using a wild species (Davis and Yu 1997).

Molecular markers have been adopted for genetic mapping because they are numerous, and are not influenced by the environment. Random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990; Welsh and McClelland 1990), through polymerase chain reaction (PCR), demonstrated that single primers of arbitrary nucleotide sequence could be used to amplify segments of genomic DNA from a variety of species.

RAPD markers have been used for genetic mapping, plant and animal breeding, for DNA fingerprinting, population genetics, and identification and isolation of chromosome-specific DNA fragments. Even though RAPD markers are mainly dominant markers and the reproducibility between different laboratories is problematical at times, they are a useful and powerful tool. RAPD markers are being used to map a wide variety of plant genomes such as bean (Haley et al. 1993; Adam-Blondon et al. 1994), blueberry (Rowland and Levi 1994), Brassica (Delourme et al. 1994; Ferreira et al. 1995), potato (Barker et al. 1994), rice (Nair et al. 1995), tomato (Chunwongse et al. 1994; Van de Beek et al. 1994), and trees and conifers (Binelli and Bucci 1994; Keil and Griffin 1994; Benet et al. 1995).

By utilising the diploid strawberry species, *Fragaria vesca* (2n = 2x = 14), genetic linkage mapping should be more feasible due to the simpler genomic structure. The objective of the present study was the construction of a genetic linkage map of a diploid strawberry cultivar, *F. vesca*.

Materials and methods

Plant material

Linkage within the diploid *F. vesca* genome was assessed through a segregating F_1 population of 55 plants from a cross between a single plant (as

female) from inbred 'Alpine' cultivar 'Yellow Wonder' and a single plant (as male) from a highly heterozygous, wild accession FRA 364 (obtained from USDA National Clonal Germplasm Repository, Corvallis, OR, USA). FRA 364 was obtained as a runner clone and was described on the Germplasm historical information sheet as a *F. vesca* clone collected from the wild in California, USA.

DNA Isolation

DNA was isolated from young (partially expanded) leaves using a modification of the method of Torres et al. (1994). Tissue samples were collected into 1.5 ml eppendorf tubes and kept on ice until DNA was isolated. Three to five small leaflets per plant (0.08 - 1.0 grams fresh weight) were ground to a fine powder in liquid nitrogen using a mortar and pestle. The resulting powder was immediately mixed with 2% extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB (Sigma Chemical Co., MO); and 0.4% β-mercaptoethanol, added just before use) in the mortar, then transferred to a 1.5 ml eppendorf tube containing 100 μl of chloroform/isoamylalcohol (24:1). Tubes were vortexed briefly, incubated in a 65°C water bath for 30 min to one hour, then allowed to cool to room temperature. After addition of 0.8 - 1.0 ml of $CHCl_3$/isoamylalcohol per tube, each tube was shaken vigorously to form an emulsion, then centrifuged in a eppendorf at maximum speed (14,000 x g) for 5 min. The aqueous phase (top layer) was then transferred to a clean eppendorf tube, and was re-extracted with $CHCl_3$/isoamylalcohol if it was still cloudy instead of clear. When the aqueous phase was clear the DNA was precipitated by addition of 1.0 ml of ice cold 95% ethanol. Tubes were kept on ice and not mixed until the DNA had begun to precipitate into the alcohol phase, after which the tubes were gently inverted three to four times, placed back on ice for 10 min, then centrifuged at 14,000 x g for 5 min. The pelleted DNA was washed in 70% ethanol. After the final wash treatment tubes containing the DNA were placed in a speed vac and spun under vacuum at maximum speed for 5 min to dry the DNA pellet. Each dried DNA pellet was resuspended in 25 μl of 10:1 Tris:EDTA (TE, pH 8.0). After quantifying the resuspended DNA using a Bio Rad fluorometer and/or an UV spectrometer, the DNA was diluted to a working solution of 40 ng/μl by adding the appropriate amount of dH_20 containing 10 mg/ml of RNAse A (5 prime → 3 prime Inc. Boulder, CO). DNA samples were RNased at 37°C for 60 min.

Polymerase Chain Reaction Procedures

RAPD primers were obtained from the University of British Columbia (Vancouver, Canada) and from Operon technology (Almalda, CA). Each 25 µl PCR amplification mixutre contained 0.5 units *Taq* polymerase (Promega Corp., Madison, WI: Mg-free) contained 2.5 µl 10X buffer (Promega Corp., Madison, WI: Mg-free), 3.5 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 µM of primer, and 50 ng of plant genomic DNA. All amplifications were performed in a Perkin Elmer Cetus DNA 480 Thermal Cycler programmed as follows: 50 s at 94°C, a slow 1.5 min ramp to 34°C, for 2 min and a 45 s ramp to 72°C for 2 min for 40 cycles then held at 72°C for 7 min before cooling to 4°C. PCR amplification products were resolved by electrophoresis in a 2.0% TBE agarose gel (1:1 NuSieve GTG:Agarose) and photographed using a Polaroid Type 55 film. The polymorphisms used for mapping purposes were those bands which were present but heterozygous (+/-) in the male parent, FRA 364, and absent (-/-) in 'Yellow Wonder' parent.

Protein Extraction, Isozyme Gel Electrophoresis and Fruit Coloured.

Two isozymes systems, phosphoglucoisomerase (PGI) and shikimate dehydrogenase (SKDH), were assessed for the parental and the F_1 plants. The morphological fruit colour (INFC) locus was scored as red or yellow coloured fruit in the progenies. The allele symbol C (red) and c (white) were assigned by Brown and Wareing (1965). Red is dominant over white (yellow) fruit colour. PGI and SKDH electrophoresis and staining were as described by Chiang et al. (1987) and Bringhurst et al. (1981).

Detection of Linkage

The program JoinMap® version 2.0 (Stam 1993; Stam and Van Ooijen 1995) was used to construct a genetic map of the markers. Linkage groups were obtained with a threshold of 3.5 LOD. The mapping function used was Kosambi's.

Results

The 19 RAPD primers chosen for the mapping project (Table 1) gave the most distinct and reproducible banding patterns among the initial 117 RAPD primers tested. Each of the 19 primers amplified between 10 - 30 bands and detected 1 - 8 polymorphisms between the parents. The segregation in the F_1

progeny was based entirely upon the markers heterozygous in the FRA 364 parental plant. Polymorphisms that were homozygous in both parents would not segregate until the F_2 generation.

Table 1: RAPD primers used for the construction of a map in the diploid strawberry.

Primer #	Sequence
BC 17	CCTGGGCCTC
BC 84	GGGCGCGAGT
BC 85	GTGCTCGTGC
BC 102	GGTGGGGACT
BC 103	GTGACGCCGC
BC 104	GGGCAATGAT
BC 105	CTCGGGTGGG
BC 106	CGTCTGCCCG
BC 122	GTAGACGAGC
BC 123	GTCTTTCAGG
BC 131	GAAACAGCGT
BC 186	GTGCGTCGCT
BC 188	GCTGGACATC
BC 190	AGAATCCGCC
BC 194	AGGACGTGCC
OPA 2	TGCCGAGCTG
OPA 4	AATCGGGCTG
OPA 5	AGGGGTCTTG
OPA 10	GTGATCGCAG

BC primers are from The University of British Columbia, Biotechnology Laboratory, Room 237-Wesbrook Building, 6174 University Boulevard, Vancouver, B.C. Canada V6T 1W5.

Analyses of segregation patterns of the 55 F_1 progeny resulted in the identification of one morphological, two isozyme and 70 RAPD markers. The RAPD markers were labelled according to primer number followed by a letter to distinguish the particular band. Of the 73 markers, 42 fit a 1:1 segregation ratio, while 31 differed significantly from the expected ratio (Table 2).

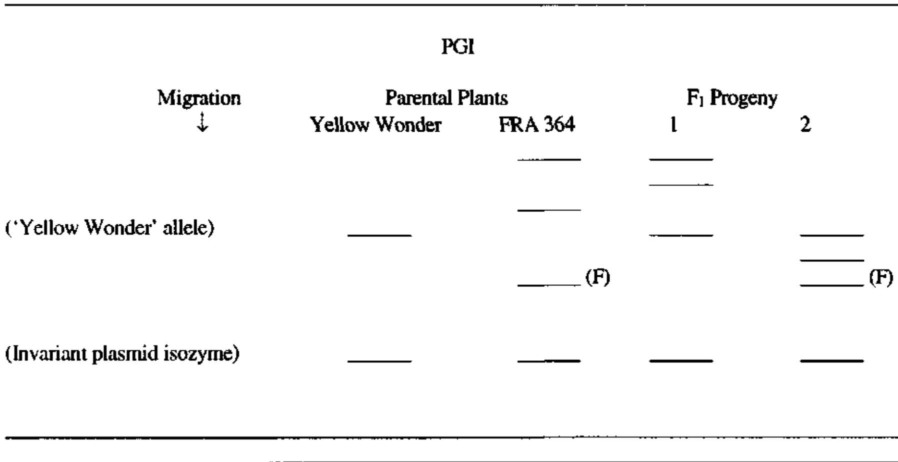
Table 2. Segregation distortion deviating from the 1:1 ratio of the 55 F_1 plants from the cross 'Yellow Wonder' x FRA 364 of the diploid strawberry. The symbol (*) indicates values significant at the P<0.01.

Marker	χ^2	Ratio	Marker	χ^2	Ratio	Marker	χ^2	Ratio
Group 1			*Group* 2			*Group* 3		
OPA 4C	0.0	27 : 26	BC 17B	4.1	20 : 35	BC 85C	19.0*	43 : 11
BC 188B	1.5	22 : 31	BC 102F	5.3	19 : 36	OPA 10B	30.6*	48 : 7
PGI 2	1.2	23 : 31	OPA 2A	0.5	25 : 30	BC 104E	19.8*	44 : 11
OPA 2G	4.1	20 : 35	OPA 5B	1.5	32 : 23	OPA 2H	22.3*	45 : 10
OPA 10F	8.0*	17 : 38	BC 188A	0.9	23 : 30	BC 186A	24.0*	45 : 9
BC 102E	0.9	24 : 31	OPA 2B	4.1	35 : 20	OPA 2E	24.9*	46 : 9
BC 85B	3.6	20 : 34	BC 105C	5.3	19 : 36	BC 105D	24.9*	46 : 9
BC 103D	3.1	21 : 34	BC 17D	5.3	19 : 36	OPA 2C	24.9*	46 : 9
BC 104B	4.1	35 : 20	BC 106B	5.3	19 : 36	BC 194C	24.9*	46 : 9
OPA 4A	4.3	19 : 34	BC 131B	3.1	21 : 34	BC 106A	24.9*	46 : 9
OPA 4B	4.3	19 : 34	BC 190C	8.0*	17 : 38	BC 122A	24.9*	46 : 9
BC 123A	7.4*	17 : 37	BC 186B	6.0	18 : 36	OPA 10E	24.9*	46 : 9
OPA 10C	8.0*	17 : 38	BC 102A	11.4*	15 : 40	OPA 2I	24.9*	46 : 9
OPA 5A	9.6*	16 : 39	BC 84A	8.0*	17 : 28	BC 190E	22.3*	45 : 10
BC 102C	4.1	20 : 35	BC 103A	4.1	20 : 35	BC 17E	24.9*	46 : 9
BC 103F	2.2	22 : 33	BC 188D	0.3	25 : 29	OPA 2F	19.8*	44 : 11
BC 122D	1.5	23 : 32	BC 194D	0.9	24 : 31	BC 188C	24.0*	45 : 9
Group 4			*Group 5*			*Unlinked*		
BC 194B	8.0*	17 : 38	BC 102B	0.0	27 : 28	BC 84B	13.3*	14 : 41
BC 131A	0.9	24 : 31	INFC	4.9	13 : 27	BC 103B	0.5	25 : 30
BC 102D	4.1	20 : 35	SKDH	0.7	30 : 24	BC 104A	8.0*	38 : 17
OPA 10D	3.1	21 : 34	BC 103E	0.5	30 : 25	BC 190A	17.5*	43 : 12
BC 84C	2.2	22 : 33	BC 84D	0.0	28 : 27	BC 194A	0.2	26 : 29
BC 103C	0.2	29 : 26	BC 190D	0.0	27 : 28	OPA 2D	13.3*	14 : 41
			BC 105B	1.5	23 : 32	OPA 2J	22.3*	45 : 10
			BC 104D	1.5	23 : 32	OPA 10A	33.6*	49 : 6

Band patterns and segregation data obtained for the isozymes, phosphoglucoisomerase (PGI) and shikimate dehydrogenase (SKDH) were as expected from past studies (Arulsekar et al. 1981a, 1981b). The PGI isozyme in 'Yellow Wonder' had a two-banded pattern, and in FRA 364 it had a four-banded pattern (Fig 1). The bottom PGI band seen in parentals and all the F_1 plants was evidently the invariant plastid isozyme as described by Arulsekar

et al. (1981a) and Arulsekar and Bringhurst (1981, 1983). The remaining PGI bands observed in F_1 progeny were either classified as slow or fast and segregated approximately 1:1. FRA 364 was therefore heterozygous for the PGI isozyme locus, while 'Yellow Wonder' was homozygous. The slow band pattern had the upper PGI band from FRA 364, a middle heteroduplex band, and a faster ('Yellow Wonder') band. The fast band pattern, in the progeny, appeared as a close triplet band with the middle band being the heteroduplex band, and the lower band being the FRA 364 fast band (Fig 1).

(a)



(b)

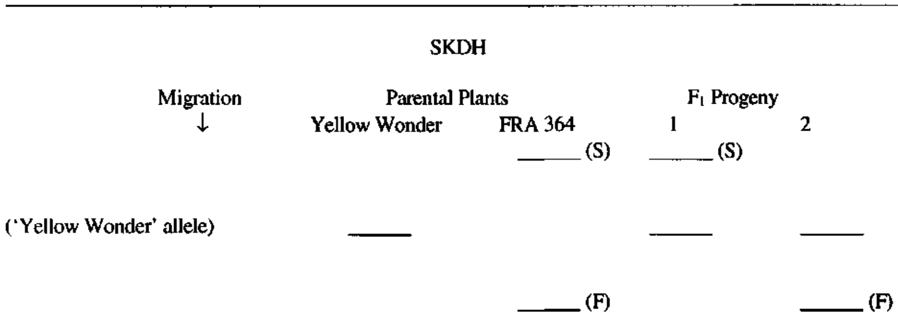


Figure 1. Isozyme analysis of PGI (a) and SKDH (b) banding patterns. Crossing parents are 'Yellow Wonder' x FRA 364. Band segregation in the progeny is based on a slow (S) versus fast (F) alleles derived from FRA 364.

The band pattern for SKDH in the parental plants was either a single band for 'Yellow Wonder' or two bands for FRA 364 (Fig 1). F_1 plants were classified as either fast or slow depending on which one of the FRA 364 band types they inherited. Thus, FRA 364 is heterozygous for SKDH, and the F_1 population was segregating with respect to the FRA 364 alleles.

A linear map order was determined for the markers using the program JoinMap (Stam 1993; Stam and Van Ooijen 1995) (Fig 2). Of the 73 markers identified, 65 markers were shown by the analysis to be linked with a minimum of 3.5 LOD. These markers were classified into five distinct linkage groups containing at least six or more markers (Fig 2). Eight RAPD markers could not be identified as linked to any of the groups. These unlinked markers may represent the two missing linkage groups.

Linkage group 1 contains 16 RAPD markers and the PGI2 isozyme, while in linkage group 2 and group 3 there are 17 RAPD markers. Linkage group 4 has six RAPD markers, while linkage group 5 contains 6 RAPD markers, the SKDH isozyme and the INFC morphological marker. The INFC fruit colour locus is tightly linked to the SKDH isozyme and to RAPD BC 103E marker, at 3 cM. The SKDH isozyme and BC 103E appear to be completely linked to each other (Fig 2).

Of the 31 RAPD markers in the F_1 progeny that significantly deviated from the expected 1:1 ratio ($P < 0.05$) only six did not map to a specific linkage group (Table 2). Seventeen of the deviating markers, mapped to linkage group 3. The other markers that deviated significantly mapped to linkage groups 1, 2 and 4. No markers in linkage group 5 deviated significantly from the 1:1 ratio (Table 2).

When RAPD OPA 2G was removed from the analysis then six linkage groups of four or more markers were obtained; by linkage group 1 becoming two separate groups. The sixth group consisted of OPA 4C, BC 188B, PGI-2, and OPA 2D, while the remaining markers in linkage group 1 remained the same.

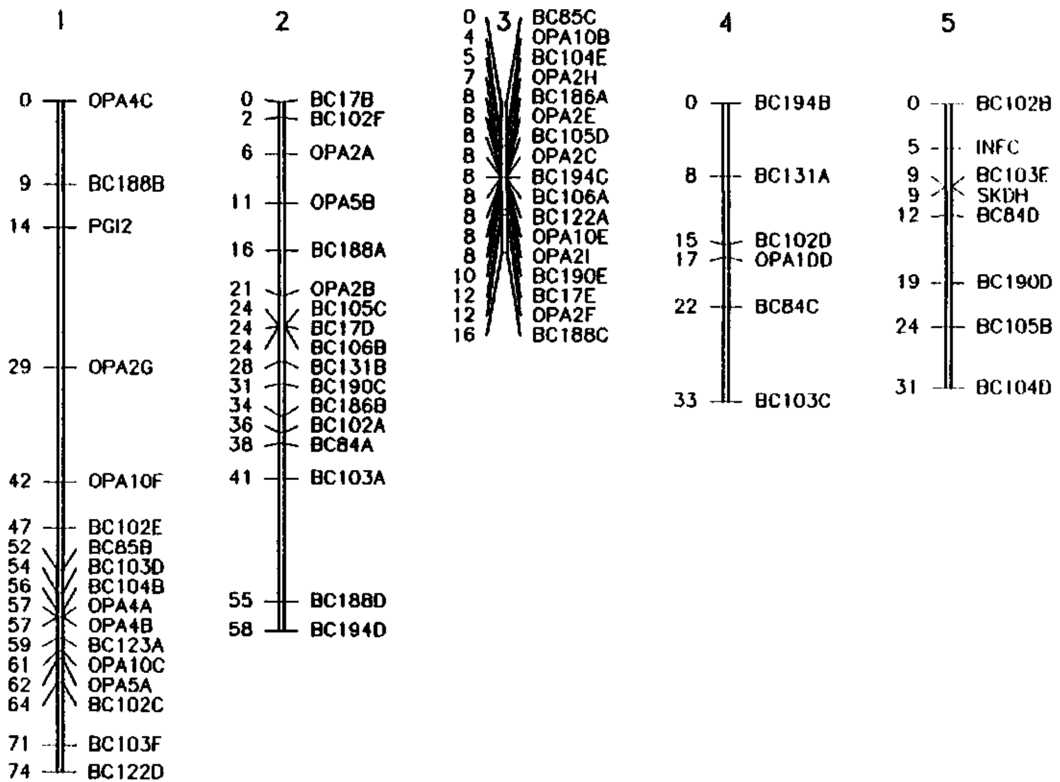


Figure 2. Linkage map of the 55 F_1 plants from the cross 'Yellow Wonder' x FRA 364 of the diploid strawberry. Map distances, written on the left, are in centiMorgans. The order and distances of the loci were derived from multiple linkage analysis using the JoinMap® 2.0 software programme with a threshold of 3.5 LOD for determining linkage and using the Kosambi function (Stam 1993; Stam and Ooijen 1995).

Discussion

The F_1 population used in the linkage study behaved as a testcross population in that one parent (FRA 364) was heterozygous and the other parent ('Yellow Wonder') was homozygous, and recessive with respect to the segregating RAPD markers. Therefore, a 1:1 segregation ratio was expected for both the dominant RAPD and codominant isozyme markers in the F_1 population. Thirty one out of the 73 markers deviated significantly from the expected 1:1 ratio. Such deviations also occur in both isozyme and RFLP analysis (Edwards et al. 1987; Tulsieram et al. 1991; Carlson et al. 1991). The small progeny size may account for random deviations from the expected 1:1 ratio, or gametic selection may be a factor. For mapping purposes some researchers discard molecular markers that segregate significantly differently from the expected ratio (Reiter et al. 1992); however, because all but three of the deviating markers were linked to at least one other marker they were included in the mapping study.

The markers analysed could be compiled into 5 linkage groups to determine the possible linear orders of the markers. Various interpretations of the data may be due to the complications of the male parent (FRA 364). FRA 364 was collected by Bringhurst in California and was originally classified as *F. vesca*, however this classifcation is now questionable. The inability to construct a complete map of the seven chromosomes and from using the plant in crosses (data not shown) has raised some doubts of the purity of the genome. We now believed that it is a hybrid between *F. vesca* and *F. viridis* species. Problems arise in that we cannot determine which genome the markers are on since no physical or cytological map are available for the diploid strawberry. However, the data obtained is still useful in that we could create a partial map of the strawberry and confirmed the possibility of future mapping of agronomic traits of interest.

The dimeric isozyme pattern of PGI as well as the invariant plastid band was present as described by Arulsekar and Bringhurst (1983). Previously, they showed PGI to be either a two-banded or four-banded pattern for *F. vesca*. In this study, PGI was classified into linkage group 1. Another relevant observation of the strawberry map includes the incorporation of the isozyme SKDH locus into linkage group 5 where it is tightly linked to RAPD BC 103E (Fig 2). Previously, Williamson et al. (1995) showed that SKDH was linked to the morphological INFC locus. Molecular markers linked to such traits can be used as gene tags to facilitate the selection of plants with desirable genotypes.

Because of its genetic similarities to the octoploid strawberry, a linkage map of the diploid strawberry can be useful in the genetic mapping of

its horticulturally important octoploid relative, *F* x *ananassa*. The known genomic homology between the diploid and the octoploid (Senanayake and Bringhurst 1967; Arulsekar et al. 1981; Bringhurst 1990) can be expected to extend to the linear order of genes on chromosomes. Selection procedures for octoploid traits may also be possible through the utilisation of gene tags identified at the diploid level.

An *F. vesca* linkage map will have various uses such as to determine evolutionary homology between the diploid versus the octoploid strawberry genomes or between strawberry and other members of the *Rosaceae* family. Strategies for map-based gene cloning would be applicable in *F. vesca* because of the small genome size. The project described here is a significant first step towards the construction of a strawberry genetic linkage map that will be a valuable tool to further develop many areas of strawberry research.

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Identification of RAPD markers linked to a *Phytophthora fragariae* resistance gene (*Rpf1*) in the cultivated strawberry

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Abstract

Bulked segregant analysis (BSA) was used to identify seven random amplified polymorphic DNA (RAPD) markers linked to the *Rpf1* gene. *Rpf1* confers resistance to *Phytophthora fragariae* var. *fragariae*, the causal agent of red stele root rot in *Fragaria* spp.. The bulked DNA's represented subsets of a F_1 population obtained from the cross Md683 x Senga Sengana which consisted of 60 plants and segregated in a 1:1 ratio for resistance or susceptibility to race 2.3.4 isolate NS2 of *P. fragariae*. Seven markers were shown to be linked to *Rpf1* and were generated from four primers; five were in coupling phase and two in repulsion phase to the gene. A linkage map of this resistance gene region was generated using JoinMap 2.0™. The manner in which *Rpf1* and the linked markers cosegregated indicated that they are inherited disomicly. These markers could enable gene pyramiding and marker-assisted selection of resistance genes in strawberry breeding programmes.

Key words red stele, linkage-analysis, *Fragaria* x *ananassa*, bulked-segregant-analysis (BSA), resistance-gene mapping

Introduction

The cultivation of strawberries (*Fragaria* x *ananassa*) is limited by several diseases, including red stele (red core) root rot caused by the fungus *Phytophthora fragariae* var. *fragariae* (Hickman 1940). Symptoms of this disease are dwarfism, wilting, and reddening of the stele. Chemical treatment such as fumigation with methylbromide and chloropicrin helps to reduce the inoculum potential in the soil but is hazardous to the environment.

Resistance to *P. fragariae* has long been assumed to be polygenically inherited (Stembridge and Scott 1959; Scott et al. 1984) but Van de Weg (1989, 1997) found evidence that red stele resistance in strawberry and the corresponding avirulence in *P. fragariae* interact according to a gene-for-gene system. At least five race specific plant resistance genes and corresponding avirulence genes are believed to exist (Van de Weg 1997). The dominant *Rpf1* resistance gene was shown to segregate monogenically (Van de Weg et al. submitted).

One of the main goals of crop breeding programmes is to combine excellent horticultural characteristics with high levels of resistance to the various pathogens. Genes conferring resistance to different races of *P. fragariae* are known to be present in some modern strawberry cultivars (Maas et al. 1989; Nickerson and Maas 1991; Kennedy and Duncan 1993; Van de Weg 1997). *Rpf1* confers resistance to at least 16 races, including the American isolates A1, A2, A3, A4, A6, A9, A10 and the Nova Scotian isolates NS1 and NS2 (Nickerson and Murray 1993; Van de Weg 1997). In the breeding of the strawberry, epistatic interactions among resistance genes creates problems when screening is done by classical methods for multiple gene resistance (Van de Weg 1989). This reduces the efficiency of disease tests and slows down the development of new cultivars. This problem can possibly be solved by indirect selection using molecular markers linked to individual resistance genes.

Genetic maps defining linkages between molecular markers and genes of interest are useful tools for plant breeders. Marker-assisted selection has shown to be a viable method for the improvement of disease and insect pest resistance. Different types of molecular markers have been employed, such as random amplified polymorphic markers (RAPD) and restriction fragment length polymorphism (RFLP) markers, in this context. RAPD markers (Williams et al. 1990; Welsh and McClelland 1990) are particularly easily and economically assayed, and they segregate in Mendelian fashion. They have been used in various crop species to map and tag resistance genes (Barua et al. 1993; Chunwongse et al. 1994; Paran and Michelmore 1993; Poulson et al. 1995; Van der Beek et al. 1994).

Mapping in the cultivated strawberry is complicated due to its octoploid (2n=8x=56) genome structure. However, when a trait is controlled by a single dominant gene that segregates in a disomic fashion, it is possible to identify linked molecular markers. Bulked segregant analysis (BSA) (Michelmore et al. 1991) is the most efficient method of identifying markers linked to single genes of interest. This approach was used to tag disease resistance genes in tomato (Van der Beek et al. 1994), common bean (Haley et al. 1993), lettuce (Paran and Michelmore 1993), potato (Pineda et al. 1993) and barley (Poulson et al. 1995). In this report, application of BSA was successfully applied to screen a strawberry population segregating 1:1 for the *Rpf1* gene and RAPD markers closely linked to and flanking this resistance gene were identified.

Material and Methods

Plant material

From the cross of two strawberry genotypes, Md683 (*Rpf1*; resistant) and Senga Sengana (*rpf1*; susceptible), 63 F_1 progeny plants were obtained. The F_1 plants were grown and runner-propagated in a greenhouse and previously characterised as resistant or susceptible to the virulent race 2.3.4 isolate NS2-25 (Nickerson and Murray 1993) of *P. fragariae* as reported by Van de Weg et al. (submitted). Two severely diseased and one symptomless plant died prior to molecular analysis. The distribution of the average disease rating among the remaining 60 plants is summarised in Table 1. All plant material was developed and maintained at CPRO-DLO, The Netherlands.

DNA isolation

DNA was isolated using a modification of the method of Torres et al. (1993). Three to five young (partially expanded) leaves per plant (0.08 - 1.0 grams fresh weight) were ground to a fine powder in liquid nitrogen using a mortar and pestle. In a mortar, 1 ml of a 2% CTAB buffer, (100 mM Tris-HCL, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% Hexa-decyl-tri-methyl-ammonium bromide, Sigma Chemical Co., MO) and 0.4% ß-mercaptoethanol was added just before use, mixed to a slurry and then transferred to a 1.5 ml microfuge tube containing 100 µl chloroform/isoamylalcohol (24:1). Samples were vortexed briefly then incubated at 65°C for 45-60 min, then cooled to room temperature. Samples were shaken vigorously to form an emulsion then centrifuged 14,000 x g for 5 min. The aqueous phase was transferred to a new 1.5 ml eppendorf tube and the DNA was precipitated by the addition of 1.0 ml

ice cold 95% ethanol. Sample tubes were gently inverted and placed on ice for 10 min, then centrifuged at 14,000 x g for 5 min. The pelleted DNA was washed in 70% ethanol, dried and resuspended in 25 µl TE, pH 8.0. To each sample, 10 mg/ml of RNAse A (5 prime → 3 prime Inc. Boulder, CO) was added and heated to 37°C for 60 min. DNA was quantified and diluted to a working solution of 50 ng/ml.

PCR conditions

RAPD primers were obtained from Operon Technology (Alameda, CA.; kits A-Z, AA, and AB; 20 primers per kit) and the University of British Columbia (Vancouver, Canada; numbers: 2, 76, 84, 88, 89 102-106, 126, 131, 190, 192, 196, 200). Each 25 ml amplification reaction contained 50 ng of genomic DNA as template. Amplification reactions contained 2.5 µl of 10X buffer (100 mM Tris-HCl, pH 9.0 (25°C), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatine and 1% Triton X-100), 100 mM of each dNTP, 30 ng primer and 0.25 U Super *Taq* polymerase (SphaeroQ, Leiden NL) overlaid with sterile mineral oil. Amplifications were performed in a Perkin Elmer Cetus DNA 480 Thermal Cycler or a Hybaid Thermal Cycler and programmed as follows: 50 sec at 94°C, a slow 1.5 min ramp to 34°C, for 2 min and a 45 sec ramp to 72°C for 2 min for 40 cycles. The reaction was then held at 72°C for 7 min before cooling to 4°C or room temperature. Amplification products were resolved by electrophoresis in a 2.0% TBE agarose gel.

Bulked Segregant Analysis

DNA of nine resistant (R) and ten susceptible (S) plants composed the respective R and S bulks. A total of 576 RAPD primers were tested on these bulks. Each primer that detected polymorphisms was repeated twice more, then tested on the 19 individuals comprising the bulks. Putatively linked markers were then tested on the entire mapping population of 60 plants. Markers with confirmed linkage to *Rpf1* were retested twice at the population level to confirm their reproducibility.

Linkage map of the *Rpf1* region

Map positions of the RAPD markers and the *Rpf1* gene were calculated with JoinMap 2.0™ (Stam 1993; Stam and Van Ooijen 1995) with a minimum LOD of 3 using the Kosambi function.

Table 1 Frequency distribution of average disease ratings for *Phytophthora fragariae* of 60 F_1 progeny plants from the cross Md683 x Senga Sengana. Disease severity was scored on a scale of 0 to 6; a 0 indicated no infection while a 6 indicated 75 to 100% infection of the roots. Averages are the mean of four replicate scores. This table is adapted from Van de Weg et al. (submitted).

Average Disease Index	% Root Infection	Number of Plants
0	0	5
0-1	4>RI>0	12
1-2	10>RI>4	12
2-3	25>RI>10	0
3-4	50>RI>25	1
4-5	75>RI>50	3
5-6	100>RI>75	27
Total		60

Results

Screening of RAPD polymorphisms by BSA.

From the 576 primers tested, 42 generated 52 putative polymorphisms. One primer detected three polymorphisms, four primers detected two, and 37 primers detected one polymorphism each. Most of these polymorphisms were stabily reproducible. However, when the nine 'R' and ten 'S' plants of the bulks were tested as individual DNA samples, segregation within the 'R' and 'S' bulks was observed for 35 out of the 52 initially identified polymorphic bands. These 35 polymorphisms resulted from unequal segregation of unlinked markers, possibly due to sampling error. Segregation pattern of 2:7, 2:8, and 3:7 were common. The analysis was continued with markers for which the bulks showed at most two recombinants. Seventeen polymorphisms produced from 14 primers were tested on the entire population. Seven of these were shown to be linked to *Rpf1* and were produced from four primers of which the sequences are indicated in Table 2. These seven markers were present in Md683 and not in Senga Sengana.

Table 2 Nucleotide sequences of four primers amplifying RAPD markers for *Rpf1* in the F_1 progeny from the cross Md683 x Senga Sengana.

Primers	5′ Sequence 3′
OPC-08	TGGACCGGTG
OPO-08	CCTCCAGTGT
OPO-16	TCGGCGGTTC
OPV-02	AGTCACTCCC

Analysis of the RAPD markers.

Five of the seven markers segregated in coupling phase to *Rpf1*, while two segregated in repulsion phase (Fig. 1). Primers OPC-08 and OPV-02 each generated one marker and primer OPO-08 yielded two markers linked to *Rpf1* (Fig. 1). The OPO-08 markers have a relatively high molecular weight (1650 bp for OPO-08A and 1800 for OPO-08F) and are difficult to score due to a bright monomorphic band of 1700 bp (results not shown). Primer OPO-16 detected three markers at the same position to *Rpf1*. Two of them, OPO-16A and OPO-16B, are in coupling phase to *Rpf1* and were faint bands. The third marker, OPO-16C, is in repulsion phase to *Rpf1* and was an exceptionally bright band (Fig. 2). Sequence analysis will indicate if the three OPO-16 markers are alternate alleles of the same locus or of two closely linked loci. The number of recombinants between the markers of Fig. 1 are: from top to bottom, 7, 2, 1, 2, 0, 0, and 4. These numbers include two double crossing-overs; one between the OPO-16 markers and respectively *Rpf1* and OPV-02D, and the other between OPO-08F and respectively OPO-08A and OPC8D.

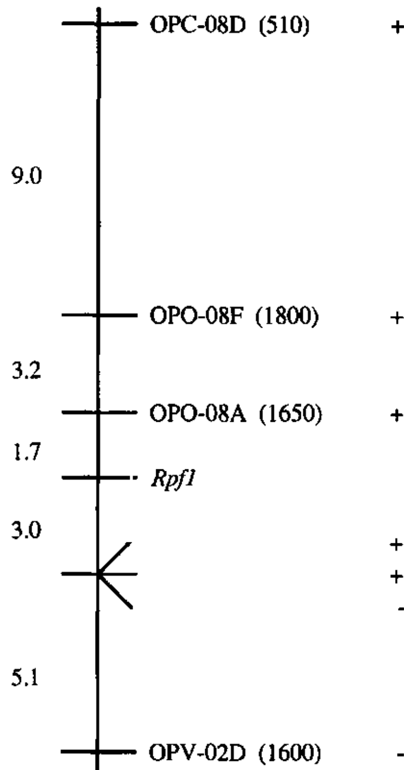

Figure 1. Linkage map of the region surrounding the *Rpf1* resistance gene and 7 RAPD DNA markers, in the F_1 of the cross Md683 x Senga Sengana of the cultivated strawberry. Map distances, written on the left, are in centiMorgans. The fragment size of the marker is given in parentheses. The phase of each marker is indicated by either a (+), coupling phase, or by a (-), repulsion phase.

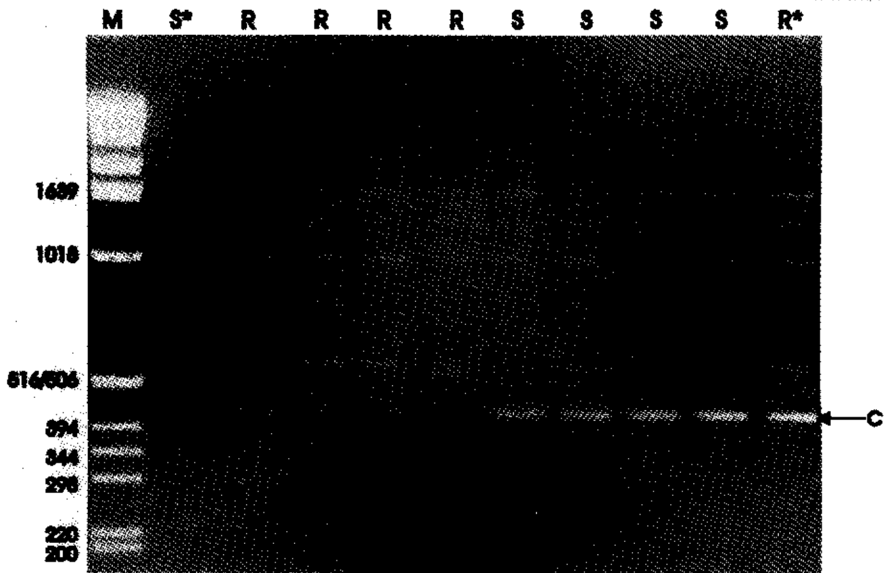

Figure 2. Individuals composing the resistant and susceptible bulks to *P. fragariae* isolate NS2-25 as screened with the RAPD primer OPO-16. Four resistant (R) and four susceptible (S) F_1 plants and the two parental genotypes were scored for the presence of the polymorphic markers indicated by the arrows. Parental plants are indicated by R* (Md683) and S* (Senga Sengana). Markers OPO-16A, B are in coupling phase while OPO-16C is in repulsion to the *Rpf1* resistance allele (Fig 1).

Discussion

The cluster of seven RAPD markers linked to *Rpf1* described here constitutes the first report of genetic linkage in the octoploid strawberry *F.* x *ananassa.* The mapping of *Rpf1* locus was possible due to the disomic behaviour of the region and the reliable classification into resistance and susceptibility of the individuals of the strawberry progeny. The results showed that BSA is an useful procedure in the strawberry to identify molecular markers for a single, dominant gene.

Disomy

The data show that at least part of the genome of the octoploid (commercial) strawberry is diploidic in nature. A 1:1 segregation in di- or polysomic inheritance can occur only in the case of a single locus when a simplex genotype is crossed with a nulliplex (recessive all chromosomes). The disomy was demonstrated by the cosegregation of two such loci, *Rpf1* and the OPO-16C RAPD marker linked to the gene. For example, OPO-16C was present in 7% (= 2/29 x 100%) of the resistant and in 100% of the susceptible progeny plants. These percentages can only be explained by disomic segregation of two linked loci at circa 3.0 cM and not by tetrasomic or octosomic inheritance. In a situation that the dominant alleles at two linked loci (e.g. *Rpf1* and the presence of OPO-16C marker) are not on the same chromosome, in repulsion, and under tetrasomic inheritance, then 33% of the resistanct plants should have the marker present when the genes are absolutely linked. Under similar condition with octosomic inheritance the marker would then have been present in 43% of the resistant progeny. The disomy is further demonstrated by the cosegregation of OPO-16C to OPO-16A. Each descendent had only one of these markers whereas with tetrasomic inheritance, 16.6% would have had each marker, and 16.6% would have none.

The data provides useful insight into the question of meiotic chromosome pairing patterns in the octoploid strawberry. Cytological data in *F.* x *ananassa* is controversial in that some studies showed primarily bivalent pairing during meiosis while others had mainly multivalent pairing (see review Galletta and Maas 1990). Further, the lack of morphological differentiation among strawberry chromosomes precludes the direct determination of whether bivalent chromosome pairing is fully preferential (autosyndetic) in the octoploid strawberry. In our results, the joint segregation pattern of OPO-16A (and -16B) versus OPO-16C can only be explained on the basis that each of the respective chromosomes are homologous and pair preferentially in Md683. If they did not pair preferentially, then some individuals in the progeny would be expected to carry either all three or none of these markers, as discussed above.

Formerly, part of the *F.* x *ananassa* genome was shown to behave as a diploid by the segregation patterns of phosphoglucoisomerase isozymes (PGI) and leucine phosphoisomerase (LAP) (Arulsekar et al. 1981; Kong and Sjulin 1993).

Indirect Selection

Md683 and its descendants have been used as founding sources of resistance in various breeding programmes. *Rpf1* is present in the cultivars created at the USDA, Beltsville such as Allstar, Surecrop, and Lester, in Canadian cultivars such as Annapolis and Cornwallis, and in selections of the Scottish and the CPRO-DLO breeding programme (Van de Weg 1997).

The establishment of markers linked to *Rpf1* confirmed the existence of this locus as a monogenic determinant of resistance to *P. fragariae*, thus supporting the proposed gene-for-gene model in the strawberry (Van de Weg 1997). Currently we are screening whether these markers are still present in modern *Rpf1* cultivars. If so, they can be utilised for indirect selection, to replace at least in part resistance tests at the plant level, and in the molecular identification of strawberry cultivars (Gidoni et al. 1994; Hancock et al. 1994; Levi et al. 1994; Parent and Pagé 1994). The only other reports of linkage in strawberry deal with isozymes linked to red vs. yellow fruit colour and to runnering vs. non-runnering in the diploid, *F. vesca* (Yu and Davis 1995; Williamson et al. 1995). There use for indirect selection in the octoploid strawberry still needs to be determined.

Strawberry breeding for disease resistance to *Verticillium* or *Collectotrichum*, and other traits of agronomic interest, would benefit from the availability of molecular markers. As shown, BSA is a useful procedure to identify such markers.

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Chapter 3

Development of a SCAR marker for the *Phytophthora fragariae* resistance gene *Rpf1* in the commercial strawberry (*Fragaria* x *ananassa*).

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Abstract

A dominant sequence characterized amplified region (SCAR) marker (linked at 3.0 cM, coupling phase) was constructed for the strawberry gene *Rpf1*, which confers resistance to the soil-borne fungus *Phytophthora fragariae* var. *fragariae*. This was accomplished by cloning and sequencing a RAPD marker fragment linked to susceptibility (*rpf1*) and converting it into a co-dominant SCAR marker. The amplified fragments from both susceptible and resistant genotypes were then cloned, sequenced and compared to the sequence of the susceptible RAPD marker fragment. Differences in homology were used to synthesize new primer sets (SCAR-R$1_{a/b}$) that amplified only in resistant plants when tested on a F_1 population segregating for the *Rpf1* gene. These primer sets can facilitate a quick and accurate test for the presence of the *Rpf1* gene in strawberry breeding programs.

Keywords: *Fragaria* x *ananassa*, RAPD markers, SCAR, disease resistance, red stele, red core

Introduction

The commercial strawberry (*Fragaria* x *ananassa* Duch.) is susceptible to red stele root rot caused by the soil-borne fungus *Phytophthora fragariae* var. *fragariae* (Hickman 1940). Symptoms of the disease are dwarfism, wilting of leaves and stem, and the stele of the roots become red in color. Soil disinfection with chemical fumigants such as methyl-bromide or chloropicrin helps to reduce the inoculum potential of *P. fragariae* but is hazardous to the environment. In most European countries and in the United States, chemicals like methyl-bromide are already banned and others are progressively being restricted due to environmental impact concerns. Another problem is that over long-term application of the chemicals the fungus may become resistant (Bollen 1972). Therefore, the development of resistant cultivars is highly desirable.

Classical breeding methods have already incorporated resistance to various diseases into the commercial strawberry. However, the ability to pyramid resistance genes and accurately screen for them is still problematic. Molecular markers linked to resistance genes might facilitate this so that fewer crosses are needed. In addition marker-assisted selection techniques can avoid the difficulties of disease resistance tests or reduce the number of plants to be tested (Tanksley et al. 1989).

Disease resistance loci have been identified in many plant species examined for breeding purposes. Genetic analysis of plant-pathogen interactions has demonstrated that resistant plants often contain single loci that specify resistance against pathogens with corresponding avirulence genes: the gene-for-gene interaction model (Flor 1971). According to the strawberry *P. fragariae* gene-for-gene model, five different resistance genes corresponded to five fungal avirulence genes exist (Van de Weg 1997).

For one of these genes, *Rpf1*, bulked segregant analysis (BSA) (Michelmore et al. 1991) was utilized to find random amplified polymorphic DNA (RAPD) markers linked to the gene in the strawberry (Haymes et al. 1997). Mapping of this gene gave molecular support to the postulate of its monogenicity and to a disease resistance response controlled by a single dominant gene (Van de Weg 1997). A problem with RAPD markers is their lack of reproducibility especially when tested in different laboratories (He et al. 1994; MacPherson et al. 1993; Weeden et al. 1992). Therefore, Paran and Michelmore (1993) described a type of marker called, sequence characterized amplified regions (SCAR) markers that are more reproducible and easier to use than RAPDs. SCAR markers are usually based upon the sequence of a RAPD band fragment by extending 10 to 14 bases to the 3′ end of the original 10-mers. In this paper, we are reporting the cloning,

and sequencing of a RAPD marker linked to *Rpf1* and its conversion into highly specific (SCAR) marker.

Materials and Methods

Plant material

A testcross between Md683 (*Rpf1*, resistant) x Senga Sengana (*rpf1*, susceptible) was performed by Van de Weg (CPRO-DLO, The Netherlands). The resulting progeny of 60 plants segregated in a 1:1 ratio for the *Rpf1* locus (Van de Weg 1997), and was utilized for the initial mapping of this gene with RAPD markers (Haymes et al. 1997).

DNA isolation/PCR amplification of RAPDs

DNA extraction and PCR amplification with RAPDs were performed according to Haymes et al. (1997). DNA from a susceptible genotype was amplified with the primer OPO-16: 5´-TCGGCGGTTC-3´ (Operon Tech, Almada, CA, USA), and separated on a 2% agarose gel (1X TBE). The PCR fragment corresponding to OPO-16C$_{(438)}$ (linked in repulsion at 3.0 cM from *Rpf1*; Haymes et al. 1997a) was cut out of the gel. The gel fragment was placed into 25 µl of 0.5X TBE for 30 min. to allow the DNA to diffuse into the buffer. RAPD-PCR re-amplification of the OPO-16C fragment was performed by using 5 µl of the DNA TBE solution, in a total volume of 50 µl. The re-amplified OPO-16C fragment was electrophoresed in a 2% TBE agarose gel and the corresponding band fragment was again excised from the gel and placed into a dialysis tube containing 500 µl 0.5X TBE, and electrophoresed for 5 min. This solution was transferred into a 1.5 ml eppendorf tube, extracted with an equal volume of phenol/chloroform (1:1), mixed, and centrifuged at 14,000 x g for 5 min. The supernatant was transferred into a new 1.5 ml eppendorf tube and 1 ml ethanol-acetate mix (100 ml 96% EtOH, 4 ml 3M NaAc (pH 5.2)) was added. The tube was gently inverted and left at room temperature for 3 min. before being centrifuged at 14,000 x g for 5 min. The DNA was washed in 70% EtOH, dried and resuspended in 30 µl sterile H_2O prior to quantification.

Cloning and sequencing of a RAPD marker

The purified DNA from RAPD marker OPO-16C$_{(438)}$ was cloned into the *Hinc* II site of plasmid pBluescript SK+ (Stratagene), and transformed to *E.*

coli DH5α according to Sambrook et al. (1989). Recombinant clones were screened for appropriately sized inserts.

Template DNA preparations and sequence reaction mixtures were done according to the ABI kit's recommended procedure. The DNA templates were sequenced from both sides on an Applied Biosystems Inc. (ABI) 373 Automated Sequencer using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing kit (ABI).

PCR reactions for RAPD and SCARs

A pair of 24 bp primers (SCAR-S) was designed on the sequence of the cloned OPO-16$C_{(438)}$ fragment (Table 1, Fig. 1). PCR reactions contained 50 ng of each of the two SCAR-S primers, 0.1 mM of each of four dNTP, 20 ng of genomic DNA, 2.5 μl 10X reaction buffer (Life Technologies), 0.75 μl 50 mM $MgCl_2$ (Life Technologies), 0.5 unit of *Taq* polymerase (Life Technologies) and 18 μl of sterilized H_2O, making a total volume of 25 μl. PCR amplification was conducted in either a Perkin Elmer Cetus Thermal Cycler 480 or a Hybaid Omnigene Cycler. PCR conditions were 94°C 3 min. followed by 35 cycles of 94°C 45 sec, 60°C 45 sec, 72°C 1.45 min., then a 7 min. extension at 72°C, and finally held at 4°C or 20°C.

Table 1. SCAR primers designed on the sequence differences between the OPO-16C RAPD marker for susceptibly and the sequenced markers for resistance from the SCAR-S primers. Map positions (Fig 1) and band fragments from which primers were designed are indicated.

			position/band fragment	
SCAR-S:	forward:	5′-TAG AAG TCT TTA AAT CGT CGT ATG-3′	41-65	(OPO-16C)
	reverse:	5′-ATT GAT GAT TAG AAA ACC CTT TGG-3′	433-410	(OPO-16C)
SCAR-R1_a:	forward:	5′-TAG AAG TCT TTA AAT CGT CGT ATG-3′	41-65	(OPO-16C)
	reverse:	5′-TGA TGC GAC ATA CAA AAA TAT TAG-3′	320-297	(Resist L)
SCAR-R1_b:	forward:	5′-ATG ACC GAA TCA AAA TAT TCT-3′	271-298	(Resist L)
	reverse:	5′-ACT AAC ACA GAC AAC CCA CCA-3′	410-390	(OPO-16C/ Resist U)

Gel analysis and sequencing of SCAR products

DNA from both susceptible and resistant genotypes was amplified using the SCAR-S primers and PCR products were run on a 3.0% TBE agarose gel. The amplified fragments were then excised from the gel and purified (as

stated previously). Sequencing reactions were set up as previously stated, except that 250 ng of purified DNA was utilized, and that the SCAR-S primers (10 μM; approximately 50 ng) were utilized instead of the forward and reverse M13 primers. New primers were developed based on the sequence of the resistant DNA fragments amplified by the SCAR-S primers (Fig. 1). Two primer sets based on sequence differences between resistant and susceptible genotypes were developed and designated as SCAR-$R1_{a/b}$ primer sets (Table 1).

PCR utilizing SCAR-R1 primers

Samples for PCR were set up as stated for the SCAR-S amplification. Samples were analyzed by electrophoresis on a 2% TBE agarose gel. The PCR buffers were optimized for each of the two SCAR-R1 primer sets. SCAR-$R1_a$: 10X buffer (100 mM Tris-HCl pH 8.8; 35 mM $MgCl_2$, 750 mM KCl); SCAR-$R1_b$ 10X buffer (100 mM Tris-HCl pH 9.2; 35 mM $MgCl_2$, 750 mM KCl) (Schoettlin et al. 1993).

Results

Development of SCAR primers based on the susceptibility allele.

The DNA fragment representing RAPD OPO-$16C_{(438)}$, linked to the susceptible allele of *Rfp1*, was cloned and sequenced (Fig. 1). Utilizing this sequence, a pair of SCAR-S primers was constructed (Table 1) that should amplify a region of approximately 396 bp in susceptible genotypes. This primer set was evaluated on the parental and the 60 segregating F_1 genotypes and amplified DNA both in the susceptible and resistant genotypes producing either a single, double or a triplet band (Fig. 2). All resistant genotypes had an band of 392 bp and one of about 345 bp, while only five resistant genotypes had also a third band of about 275 bp. All susceptible genotypes were characterized by a single band of 392 bp except for seven genotypes which have a second band (~335 bp) similar to the resistant plants. This band from susceptible genotypes was at a slightly lower molecular weight than the corresponding second band from resistant genotypes.

Both bands from two susceptible genotypes were isolated, sequenced and found to be identical to the OPO-16C sequence region between the SCAR-S primers (results not shown). From two resistant genotypes that produced a doublet band with the SCAR-S primer set, both the 392 bp and the 345 bp band were isolated and sequenced. The

sequences from both of these fragments were identical to each other except that the 345 bp band ended directly prior to the SCAR-S reverse sequence. The reverse SCAR most likely is present but we were unable to detect it in the sequence. This 345 bp fragment was not investigated any further. However, the 392 bp sequence did contain both the SCAR-S forward and reverse sequence. The lowest triplet (275 bp) band from resistant genotypes was sequenced and differences in the sequences between resistant and susceptible genotypes were noted (Fig. 1). The 275 bp sequence had the SCAR-S forward primer and ended 25 bp downstream from the SCAR-S reverse primer. Again the reverse SCAR was most likely present but we were unable to detect it. Therefore, each of these 3 fragments are about the same length in DNA sequence but have different mobility on the agarose gel.

The sequence from the 392 bp resistant band had a 99% homology to the susceptible allele marker (OPO-16C) (Fig. 1). A two bp change in the sequence and one unmatched 'T' were observed as the only differences (Fig. 1). The 275 bp band was very similar to both the susceptible and the resistant band 392 bp sequence (96% homology) (Fig. 1). This resistant fragment when compared to the OPO-16C sequence, indicated 8 unmatched bases of which 7 were part of a deletion region and a total of 6 bp changes between the OPO-16C and the lowest 275 bp sequence were observed (Fig. 1).

In a comparison of the resistant genotypes 392 bp sequence to the 275 bp sequence, a 7 bp deletion region and 5 bp changes were observed. The region of the 275 bp fragment sequence that showed a higher rate of homology discrepancies (position 284 to 320) was used for creating specific primers linked to the resistant allele.

```
           1                                                50
OPO16C     TCGGCGGTTCTACGCATTAAGATGCACTTGCATCATTAATGTAGAAGTCT
Resist U                                            •••••••••
Resist L                                            •••••••••

           51                                              100
OPO-16C    TTAAATCGTCGTATGTGAAGATCCAATGAAGACTAGAGACCCTAAATCTT
Resist U   ••••••••••••••••••••••••••••••••••••••••••••••••••
Resist L   ••••••••••••••••••••••••••••••••••••••••••••••••••

           101                                             150
OPO-16C    TTAATTTTATTTTATTTTGATCCGATCAAATTTAACGCTCATAATAAG
Resist U   ••••••••••••••••••••••••••••••••••••••••••••••••••
Resist L   ••••••••••••••••••••••••••••••••••••••••••••••••••

           151                                             200
OPO-16C    ATTACATCTTATTCGACCTTAGGTTCTTACCTTATGCATGTGTTGGAAAC
Resist U   ••••••••••••••••••••••••••••••••••••••••••••••••••
Resist L   •••••••••••••••••••••••••••••••••••••••••••••••••T

           201                                             250
OPO-16C    ACTTGAGACTA-TTTTTTAAATTTAAATTTTATTAACGGTATTAGAATCC
Resist U   •••••••••••T••••••••••••••••••••••••••••••••••••••
Resist L   •••••••••••T••••••••••••••••••••••••••••••••••••••

           251                                             300
OPO-16C    ATGCATGATAATCCGACATGATGACCGAATCAAGATATTACATATTAAAA
Resist U   •••••••••••••••••••••••••••••••••A••••••••••••••••
Resist L   •••••••••••••••••••••••••••••••••A-------•••••CT••

           301                                             344
OPO-16C    GAATTTTGTATGTCGCAGCATTTTTCCTTTGATCATTTGATGGGATAGAG
Resist U   •••••••••••••••••T••••••••••••••••••••••••••••••••
Resist L   T•T••••••••••••••T••••••••••••••••••••••••••••••••

           351                                             400
OPO-16C    CAGCGTAGTCGCGAGGTTAAAGCAGTTTCAGCTTTCTTGTGGTGGGTTGT
Resist U   ••••••••••••••••••••••••••••••••••••••••••••••••••
Resist L   •••••••••••••••••••••••••••••••••••

           401                                 438
OPO-16C    CTGTGTTAGTAACTACTAATCTTTTGGGAAACCGCCGA
Resist U   •••••••••••••••••••••••••••••••••
Resist L
```

Figure 1. Sequence homology between the susceptible OPO-16C$_{(438)}$ and the corresponding upper 392 bp (Resist U) and lowest 273 bp (Resist L) resistant bands amplified with the SCAR-S primers from Fig 2. Alignment and homology analysis was done using the multiple sequence alignment and cluster analysis. Base pair changes are indicated directly below the sequence and deletion regions are noted by the symbol (-). The original RAPD primer is underlined and in bold text.

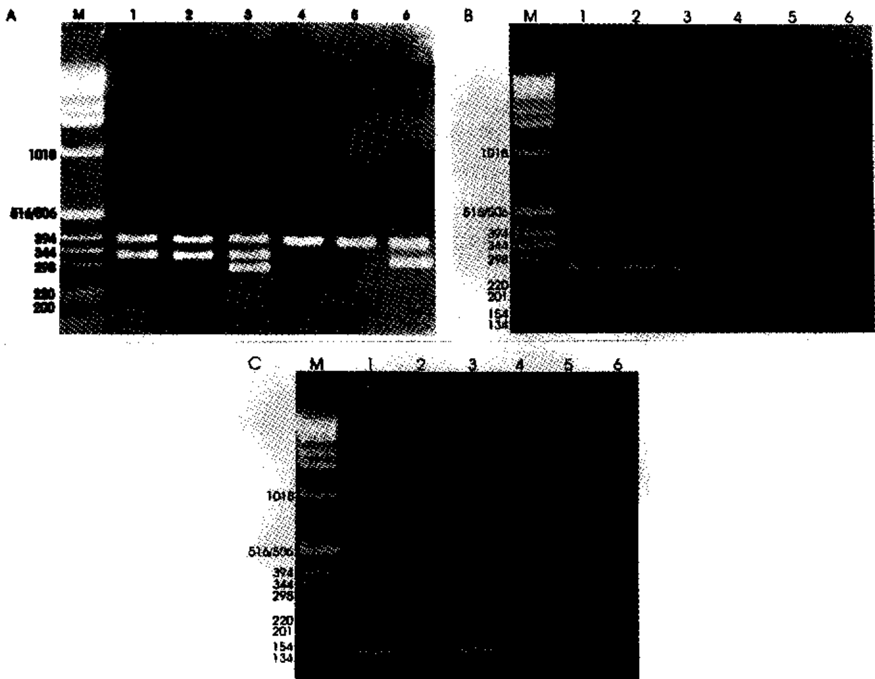

Figure 2. DNA of three resistant and three susceptible plants amplified with the SCAR primer sets. A) SCAR-S, B) SCAR-R1_a, and C) SCAR-R1_b. Amplified products were analyzed by electrophoresis on 2% TBE agarose gel; lanes 1-3 are *Rpf1* resistant plants and lanes 4-6 are *rpf1* susceptible plants to *P. fragariae* isolate NS2-25. Molecular weights (M) are in base pairs. Primer sequences are listed in Table 1.

Construction of SCAR primers to the resistant allele of *Rpf1*

Based on the deletion region observed in the 275 bp sequence, two SCAR-R1 primer sets were designed to amplify DNA from the resistant plants only. The SCAR-R1_a forward primer consisted of the original forward SCAR-S primer (Table 1). The reverse primer consisted of sequence differences at the 3′ end by including the deletion region in the construction of the primer (Table 1). For SCAR-R1_a, a band of about 280 bp was amplified in resistant genotypes only (Fig. 2b), except in the recombinants.

The SCAR-R1_b forward primer was based on the deletion region and a two bp change in the nucleotides sequence at the 3′ end (Table 1). This SCARs reverse primer started 28 bp upstream from the original RAPD primer and the expected length of the amplified region was 133 bp (Fig. 2c). The SCAR-R1_b primers were tested on the F_1 mapping population and amplified the expected fragment in resistant plants only, except in the recombinant plants.

Discussion

Genetic markers represent a useful tool for plant breeding since the presence of genes can be detected at an early stage of plant development without waiting for the phenotypic expression of the gene in the plants. In this study, a SCAR marker was originally constructed based upon a RAPD marker (OPO-16C$_{(438)}$) linked to the susceptibility allele of the *Rpf1* gene. To overcome the disadvantages associated with RAPD markers, such as irreproducibility among laboratories, we converted the OPO-16C RAPD marker into a SCAR marker (Paran and Michelmore 1993).

Various other research groups (Paran and Michelmore 1993; Kaplan et al. 1996; Xu et al. 1995) have developed codominant and dominant SCAR markers and showed that they can be useful for marker-assisted selection and high resolution mapping.

The constructed codominant SCAR-S primers amplified DNA of different molecular weights in susceptible and resistant plants as seen on the agarose gel. The multiple bands produced from this SCAR primer set may be due to amplification of a duplication region of the genome. Another possibility is that these three similar sequences from the resistant genotypes may be formed by a heteroduplex. The 392 and 275 bp fragment would be a homoduplex while the 345 bp fragment would be a heteroduplex.

Rpf1 confers resistance to at least 16 different races of the fungus (Van de Weg 1997b) and is one of the main genes for resistance in several breeding programs. The SCAR-R1$_{a/b}$ markers cosegregated completely with the OPO-16 (A, B and C) markers (Haymes et al. 1997), indicating that these primers most likely amplify the same genomic region. Given the close linkage between *Rpf1* and the SCAR-R1 markers (3.0 cM), this marker should be useful in breeding programs. *Rpf1* represents one of the major sources of genetic resistance to *P. fragariae*. In pyramiding strategies, that are considered for the development of durable disease resistance, the identification of molecular markers for each desired resistance gene is required. In our laboratory we are currently mapping two more *Rpf* genes and this should allow the application of efficient selection schemes for pyramiding red stele resistance genes in superior varieties, provided that they map to different chromosomal regions. If they do map to the same chromosomal region, then recombination frequency between them will determine whether or not they are useful.

Young and Tanksley (1989) demonstrated that marker-assisted selection during backcross programs could largely reduce both the number of required steps and the length of introgressed fragments. This selection procedure can be employed to examine whether or not introgression of a

desired trait has happened when molecular markers surround the gene. This procedure appears to be faster and easier than performing complicated tests to establish the phenotypic value of a plant . However, tight linkage of markers and trait is required to diminish the chance of recombination. In the strawberry the *Rpf1* is flanked by a SCAR at 3.0 cM and a RAPD (OPO-08A) at 1.7 cM (Haymes et al. 1997).

These SCAR-R1 primers allowed the quick screening for the presence of the *Rpf1* gene and amplified DNA only from the resistant plants in the F_1 mapping population. Whether the primers are also useful in other cultivars and genotypes remains to be established. The molecular characterization of this locus is of interest for the understanding of the mechanisms underlying specific pathogen resistance in plants.

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Chapter 4

A DNA mini-prep method suitable for a plant breeding program

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Abstract

Plant breeders need a non-destructive and inexpensive protocol to screen large numbers of plants early after sowing. Isolation of DNA represents one of the limiting steps in this process: normally a person is capable of isolating 25-50 samples per day. To speed up the DNA isolation an inexpensive, non-destructive DNA mini-preparation CTAB method was adapted for use in a marker-assisted plant breeding program. With this protocol a single individual is capable of isolating 200-250 samples per day of high quality DNA that is immediately suitable for testing by PCR. The amount of DNA isolated is sufficient for about 150 PCR amplifications. This improved protocol is achieved by eliminating time consuming and non-critical steps of a standard protocol such as extensive grinding, EtOH washing and RNAse A treatment.

Abbreviations: CTAB, hexadecyl-trimethyl-ammonium bromide; dH_20, deionized water; EtOH, ethanol; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; TE, tris-EDTA (*see Material and Methods*).

Key Words: DNA isolation, PCR, Southern blots

Introduction

The advent of the polymerase chain reaction (PCR) has opened up new avenues for genetic analysis because of its simplicity, as hundreds of DNA samples can be quickly screened. Isolation of the DNA represents one of the limiting steps in the development of a feasible method of utilizing molecular markers in breeding programs. Prerequisites for a successful DNA isolation protocol is that small amounts of plant material are needed: it needs to be rapid, reproducible, and the DNA solution must be low in phenolic compounds. A single person normally isolates about 25 to 50 DNA preparations per day. In many of the DNA isolation protocols time-consuming, multiple steps are involved. Because of this, DNA mini-prep methods were developed. Many mini-prep protocols have particular disadvantages, such as destructive to the seedlings, poorly reproducible, low DNA yield, and presence of phenolics (Brunel 1992; Langridge et al. 1991; Edwards et al. 1991; Wang et al. 1993). The method by Berthomieu and Meyer (1991) uses small leaf or root sections that are placed directly in the PCR mixture, and it is good for detecting specific markers. The protocols of Wang et al. (1993) and Berthomieu and Meyer (1991) are quick and easy but, because the DNA yield is limited, they are not useful for more extensive mapping, where several markers should be determined. Another disadvantage is the greater chance for contamination from field-grown material, and the high amounts of phenolic compounds in the DNA solution. Protocols have been developed to overcome the problem of phenolic compounds in plant tissues that could interfere with PCR amplifications (Dellaporta et al. 1983; Tai and Tanksley 1990; Couch and Fritz 1990). Even though these protocols yield large quantities of clean DNA, because they are time consuming, they are not applicable in a breeding program.

For our purposes, a DNA prep was needed that could be integrated into a marker-assisted breeding program. Seedlings needed to be tested prior to field trials without losing the plants. A successful DNA isolation protocol was developed that is suitable for a plant breeding program because it is simple, rapid, by eliminating non-essential steps; and yields high quality DNA.

Material and Methods

Reagents

ethanol-acetate solution: 96 mL EtOH with 4 mL 3M NaAc (pH 5.2)
extraction buffer: (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% Hexadecyl-trimethyl-ammonium bromide (CTAB), Sigma Chemical Co., MO) and 0.4% ß-mercaptoethanol.
TE: 10 mM Tris, 1.0 mM EDTA, pH 8.0
Chloroform/Isoamyl alcohol (24:1)
70% EtOH v/v (optional)
RNAse A (optional): 10 mg/mL

Plant Material

Strawberry (*Fragaria* x *ananassa*) leaf tissue: about 1000 two- to three-week old seedlings, about 5 cm high, grown in a greenhouse, and 50 plants from field material. Leaf material from twenty *Gladiolus* spp. were from tissue culture, while about 50 each of leek (*Allium porrum* L), onion (*Allium cepa* L), and tomato (*Lycopersicon esculentum* Mill.) were from greenhouse-grown plants; and the various apple (*Malus* spp.) genotypes came from the field.

Procedure[1,2]

- Collect leaf tissues by closing the top of an 1.5-mL eppendorf tube over a small section of a leaf to obtain samples of the same uniform size.[3]
- Grind the circular leaf tissues to a fine powder or pulp, using a hand pestle that fits into the 1.5 mL eppendorf tube, attaching the pestle to a desktop or to a hand drill. The leaf tissues were ground about 10 s by hand or 2-5 s with the power drills.[4]
- Add 250 µL of extraction buffer to each sample and then create a slurry in each eppendorf tube with either a 5-s pulse from a vortex or a few quick twists using the pestle.
- Incubated the samples for 15 to 30 min at 65°C.
- Add 100 µL of chloroform/isoamyl alcohol to each tube and vortex briefly.
- Centrifuge the samples for 3 min maximum speed, and transfer the upper aqueous layer to a new tube.
- Precipitate the DNA by adding 500 µL ethanol-acetate solution at room temperature.[5]

- Centrifuge the samples for 3 min maximum speed and discard supernatant.
- Optional step: wash the pellet with 70% EtOH.
- Dry samples in a speed vac or air dry 1 h.
- Resuspend the samples in 200 µL dH_20.
- Optional step: Treat the samples with 1 µL RNAse A for 60 min at 37°C.
- Samples could be used directly for PCR after resuspension (1 µL/25 µL reaction).

Notes:

1. This protocol can be adapted for use in Southern blotting by increasing the initial amount of leaf material to 1-3 immature leaves. The isolated DNA on average was then 10-15 µg. Only one step in the procedure needs to be changed: the amount of extraction buffer used should be increased from 250 µL to 500 µL.
2. The DNA mini-prep method was successfully used on a pure culture of a fungus of the Basidiomycota, *Pisothus tinctorius*, by collecting 50 mg mycelium from 3-week old colonies directly into a 1.5 mL tube. Fungal colonies were frozen in liquid nitrogen and ground to a powder. DNA was then isolated as stated in the presented protocol, except samples were resuspended in 50 µL dH_2O with RNAse A.
3. Plant material collected in the field can be placed directly into a cooler containing ice or into plastic bags with paper towels wetted in water.
4. The desktop drill method proved quicker and easier to grind numerous leaf samples. After each sample, the pestle was cleaned in water, and then dried.
5. It saved time if the upper aqueous layer was transferred directly to 1.5 mL eppendorf tubes pre-filled with the ethanol-acetate solution.

Results and Discussion

Marker-assisted breeding programs are limited by the number of plants from which the DNA can be isolated, a problem eliminated by the protocol presented here. Strawberry plants are high in phenolic compounds, and the tissue is very thick and fibrous; liquid nitrogen was therefore used in the extraction protocol. The other plants tested: apple, gladiolus, leek, onion and tomato are very fibrous, but the DNA isolated without liquid nitrogen proved to be very clean. It is recommended that the leaf tissue be first tested for DNA isolation without liquid nitrogen, possibly eliminating one more step in the

protocol. Various grinding methods were assessed, and the hand drill or hand pestle for the eppendorf tube was easiest to use when material was ground immediately upon collection and then stored on ice. If samples were collected first and then ground later, the desk-top drill proved to be the quickest and the easiest method. Since they are only used for a few seconds, the powered drills do not destroy the DNA, but if they are used for about 15 s or longer, the DNA may shear. Leaving out various steps, such as cleaning with 70% EtOH and RNAse A, still proved to be feasible for successful PCR amplification. More time was saved by pre-labeling tubes and transferring the DNA solution into eppendorf tubes containing ethanol-acetate for precipitation. The genome sizes for the plants tested varied greatly in size, from strawberry, which is about the size of *Arabidopsis thaliana*, to *Allium* spp. which have 20-fold larger genomes. Each of the plants isolated with this protocol yielded DNA that could be amplified by RAPD-PCR. The DNA samples amplified successfully, whether used directly after alcohol precipitation, and then resuspension in water, or if they were first washed in 70% EtOH and then digested with RNAse. These last two steps, therefore, are optional for RAPD-PCR amplification, but the steps are recommended if the DNA is to be used for Southern blotting.

The dilution range for PCR amplifications needs to be determined for each plant species. If the concentration of DNA is low, it is recommended to grind the leaf material longer till it forms a liquid pulp, and to use young leaf material. Another method of increasing the DNA yield is to lengthen the incubation of samples longer at 65°C up to 60 min.

This quick prep method when used with a marker-assisted breeding program can greatly increase the capability of selecting the desired genotype. The method is quick, efficient, inexpensive, and non-destructive of the plants.

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Chapter 5

Conservation of linkage of RAPD and SCAR markers to the *Rpf1* resistance gene for *Phytophthora fragariae* in strawberry.

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Abstract

European and North American strawberry (*Fragaria* spp.) genotypes were evaluated for the presence of random amplified polymorphic DNA (RAPD) and sequence characterised amplified region (SCAR) markers previously found to be linked to the *Rpf1* gene. This gene confers resistance to red stele root rot disease, caused by *Phytophthora fragariae* var. *fragariae*. The markers were generally present in resistant genotypes and absent in susceptible genotypes. The *Rpf1* region thus showed to be highly conserved. Therefore these RAPD and SCAR-PCR markers can be used in pedigree-analysis studies, cultivar identification and in breeding programmes for the selection of red stele (*Rpf1*) resistant genotypes.

Key words: marker-assisted selection, PCR, pedigree-analysis, RAPD, resistance gene mapping, SCAR

Introduction

In strawberry, red stele root rot caused by *Phytophthora fragariae* var. *fragariae* is one of the most destructive diseases. Five genes for resistance to *P. fragariae* have recently been described, including *Rpf1* (Van de Weg 1997). *Rpf1* is a dominant gene (Van de Weg et al. 1997b) that confers resistance to numerous races of *P. fragariae* (Kennedy and Duncan 1993; Nickerson and Murray 1993; Scheewe 1994; Van de Weg 1997).

Molecular markers may enhance plant breeding programmes through marker assisted selection. In breeding programmes the ability to quickly select resistant plants is essential to maximise efficiency. Molecular marker assisted selection can accelerate this process and examine whether or not introgression of a desired trait has happened. To be useful, these markers need to be tightly linked to the gene of interest and to be conserved in successive generations.

One type of molecular marker called randomly amplified polymorphic DNA (RAPD) (Williams et al. 1990) has been utilised in numerous species (for reviews see Devos and Gale 1992; Waugh and Powell 1992; Tingey and Del Tufo 1993). RAPD markers are difficult to reproduce in different laboratories (He et al. 1994; MacPherson et al. 1993; Weeden et al. 1992). Therefore, RAPD markers linked to horticultural characteristics have been converted into more specific and reproducible markers called, sequence characterised amplified region (SCAR) markers (Paran and Michelmore 1993). Recently, RAPD markers linked to the *Rpf1* gene of the strawberry were identified in the *Fragaria* x *ananassa* selection Md683 (Haymes et al. 1997a), and one was converted into a SCAR marker (Haymes et al. 1997b). In the present study, the degree of conservation of these RAPD and SCAR markers in European and North American strawberry genotypes and breeding selections in relation to the *Rpf1* gene was examined.

Material and methods

Plant material

Strawberry genotypes came from the CPRO-DLO strawberry collection and breeding programme, and from Dr. K. Hummer (USDA National Clonal Germplasm Repository, Corvallis, OR, USA) (see Table 1). In total 36 European and 50 North American genotypes were tested.

Assessment of the presence of the *Rpf1* gene

The assessment of the presence of the *Rpf1* resistance gene was based on analysis of the resistances of genotypes to individual isolates of the fungus in view of the gene-for-gene model (Van de Weg 1997). The analysed data were obtained from earlier reports or in resistance tests (see below). For a few genotypes no data on resistance were available. For a few genotypes, including some North American genotypes introduced prior to 1940, no data on resistance tests were available. For these we concluded that they lack *Rpf1* based on their susceptibility in naturally infested fields in the USA (Darrow 1966) and in The Netherlands (Van de Weg and Meulenbroek unpublished data), in which *Rpf1* was found to be effective (Van de Weg et al. 1997b).

Resistance tests

Resistance tests were performed at either CPRO-DLO, The Netherlands or at the USDA/ARS Fruit Laboratory, MD, USA. At CPRO-DLO, *Rpf1* genotypes were identified as such by their resistance to the North American isolate NS2 and susceptibility to one of the USA isolates A7 and A8, and to the Canadian isolates NS3 and NS4 (Van de Weg 1997). Tests were performed according to Van de Weg et al. (1997a). In short, young rooted runner plants were inoculated by dipping their roots in macerated mycelium. Next, they were potted and grown under conditions conducive for the disease. Three weeks after inoculation, plants were lifted and the disease severity of the roots was assessed on a scale of 0 (symptomless) to 6 (severely diseased). Classifications were based on four plants which were split equally between two independent resistant tests. Genotypes were classified as resistant if their average disease score was three or less, while in the same test the susceptible reference cultivar 'Blakemore' had an average disease score of at least five.

At the USDA/ARS Fruit Laboratory, *Rpf1* genotypes were identified by their resistance to isolate A3 or to a mixture of A1, A2, A3, A4, and A6. In addition some genotypes were tested with some individual isolates (A1, A2, A4, and A6) for which susceptibility indicates absence of *Rpf1*. Tests were performed according to Scott et al. (1975) and Maas et al. (1989). In short, young runner plants were inoculated by dipping their roots in macerated mycelium, and planted in a greenhouse bench filled with sand. After three months, plants were lifted and their disease severity was assessed on a scale of 1 (most roots dead) to 10 (symptomless). Genotypes were considered to be resistant if their average disease score was

considerably higher than that of a cultivar known to be highly susceptible to the disease. Each genotype-isolate combination was tested by at least four plants equally divided over four randomised replications.

Molecular markers studies

Two RAPD markers (OPC-8D; 5´-TGGACCGGTG-3´ and OPO-8A; 5´-CCTCCAGTGT-3´) and one SCAR marker (SCAR-R1$_a$: forward: 5´-TAGAAGTCTTTAAATCGTCGTATG-3´; reverse: 5´-TGATGCGACAT ACAAAAATATTAG-3´) linked to *Rpf1* (Haymes et al. 1997a, 1997b) were utilised (Fig. 1). The RAPD markers are situated on one side and the SCAR marker on the other side of the *Rpf1* locus. RAPD marker OPC-8D was located at 13.9 cM, OPO-8A at 1.7 cM, and the SCAR-R1$_a$ marker at 3.0 cM from *Rpf1* (Fig. 2). These markers are all in coupling phase to *Rpf1*. Additionally, RAPD OPO-16C (5´-TCGGCGGTTC-3´) was utilised since it is an alternate allele of SCAR-R1$_a$ in Md683, the genotype in which the markers were originally identified (Haymes et al. 1997b).

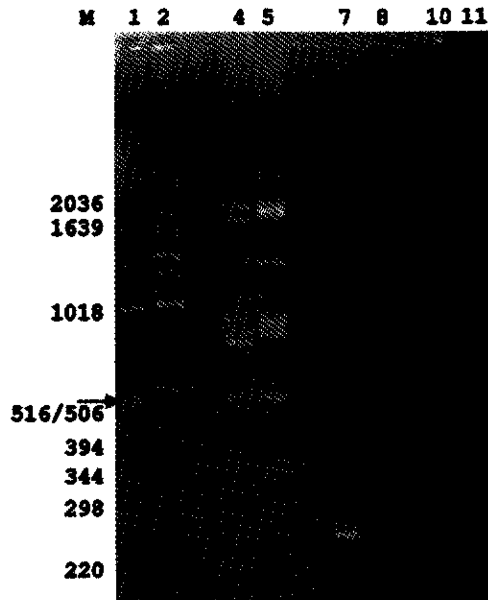

Figure 1. DNA amplification of a *Rpf1* and *rpf1* genotype as screened with the RAPD primer OPC-8 (lanes 1 and 2), OPO-8 (lanes 4 and 5), SCAR-R1$_a$ (lanes 7 and 8), and OPO-16 (10 and 11), respectively, and lanes 3, 6, and 9 are empty. The polymorphic markers OPC-8D, OPO-8A, SCAR-R1$_a$, and OPO-16C previously identified as being linked to the *Rpf1* gene are indicated by an arrow. The molecular weight (in bp) is indicated by the symbol M.

PCR conditions for RAPD and SCAR primers

DNA was isolated following the protocol of Haymes (1996). PCR amplification, electrophoresis, staining, and scoring of the bands were according to Haymes et al. (1997a, 1997b). RAPD primers were obtained from Operon Technology (Almalda, CA, USA).

Results

Correlation of the molecular markers with *Rpf1*

The results of both molecular marker studies and the plant resistance screening tests for the presence of *Rpf1* are presented in Tables 1 and 2. The three RAPD and SCAR-R1_a markers were examined on 86 genotypes of which 34 possess *Rpf1* and 52 lack *Rpf1* (Table 1, 2). RAPD OPO-8A marker, the marker nearest to *Rpf1* (Fig. 2), correctly assessed 29 out of the 34 resistant genotypes as well as all the susceptible (*rpf1*) genotypes (Table 1, 2). The divergent resistant genotypes were 'Perle de Prague' and four CPRO selections: 88218, 88275, 88310, and 89027.

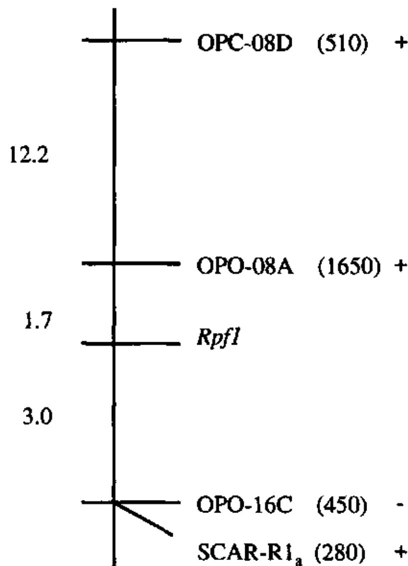

Figure 2. A RAPD and SCAR linkage map of the *Rpf1* region. Map distances, written on the left, are in centiMorgans and the marker fragment size (in bp) is given in parentheses. The phase of each marker is indicated by either a (+), coupling phase, or by a (-), repulsion phase. The original RAPD map is from Haymes et al. 1997.

The SCAR-$R1_a$ marker identified 23 of the 34 *Rpf1* genotypes correctly, as well as all *rpf1* genotypes but one (Table 1, 2). Eight of the 11 divergent *Rpf1* genotypes are inter-related and their lack of linkage can be traced back to a single crossing-over event during meiosis in parent Md683 of the cross 'Fairland' x Md683. In the resulting cultivar 'Stelemaster' *Rpf1* is no longer linked to the SCAR but to the alternate allele OPO-16C. Consequently SCAR-$R1_a$ is absent and OPO-16C is present in its descendants: 'Delite', 'Scott', 'Sunrise', 'Tribute', CPRO 88218, CPRO 88310, and CPRO 88312. The three other SCAR-$R1_a$ divergent genotypes were: 'Benton', CPRO 77191, and 'Perle de Prague'. 'Cambridge Vigour' was the only susceptible genotype which possessed the SCAR marker.

RAPD OPC-8D, the marker farthest from *Rpf1* (Fig. 2), had a total of 16 non-conforming genotypes out of 86. From the 34 *Rpf1* genotypes 11 were divergent from the known resistance ('Annapolis', 'Arking', 'Delite', 'Linn', 'Tribute', 'Perle de Prague', 'Yalova-4', 'Yalova-15', CPRO 88239, CPRO 88246, and CPRO 88275) (Table 1). From the latter five a single crossing-over event occurring in one of the parental genotypes ('Cengelköy' or an earlier ancestor) was the reason for the loss of the marker, while the absence of the marker in 'Arking' came from 'Delite'. Out of the 52 susceptible genotypes, five possess the OPC-8D marker (Table 2).

Table 1: Comparison of European and North American strawberry genotypes for the presence of four markers and the *Rpf1*-gene: all genotypes were tested for resistance to *Phytophthora fragariae*. Three markers are in coupling phase to *Rpf1* and located at 13.9 cM (OPC-8D), 1.7 cM (OPO-8A), and 3.0 cM (SCAR-$R1_a$ marker) from the gene. These RAPDs are on one side of the gene and the SCAR is on the opposite side. The fourth RAPD marker (OPO-16C), is an alternate allele of SCAR-$R1_a$, and is generally in repulsion phase to *Rpf1*.

Genotype name	Country of Origin[1]		Parental Plants ♀		Parental Plants ♂	OPO 16C	OPC 8D	OPO 8A	SCAR $R1_a$	*Rpf1*[2]
Allstar	USA	u	US4419 ((Redstar x Surecrop) x ((MD1972 x Midland) x (Redstar x MD430)))	x	MDUS3184 (NC-1768* x Surecrop)	-	+	+	+	+ a
Annapolis	CAN	u	(Micmac x Raritan)	x	Earliglow	-	-	+	+	+ a
Arking	USA	u	Cardinal	x	ARK-5431(MDUS3082 (NC-1768* x Surecrop) x Delite)	-	-	+	+	+ b
Auchinicruive-6	UK	u	Frith	x	Frith	-	+	+	+	+ c
Benton	USA	u	ORUS 2414	x	Vale	-	+	+	-	+ d
Cornwallis	CAN	u	Earliglow	x	Kent	+	+	+	+	+ a
CPRO 77191	NL	c	Guardian	x	Sivetta	+	+	+	-	+ e
CPRO 88218	NL	c	Bogota	x	Scott	+	+	-	-	+ e
CPRO 88239	NL	c	Bogota	x	Yalova-4	-	-	+	+	+ e
CPRO 88246	NL	c	((Redchief x Sivetta) x Bogota)	x	Yalova-4	-	-	+	+	+ e
CPRO 88275	NL	c	(Holiday x (Induka x Sivetta))	x	Yalova-4	-	-	-	+	+ e
CPRO 88310	NL	c	((Sivetta x Holiday) x Korona)	x	Scott	+	+	-	-	+ e
CPRO 88312	NL	c	same as CPRO 88310			+	+	+	-	+ e
CPRO 89027	NL	c	((Tamella x Redgauntlet) x MDUS2700 (Pocohantas x Stelemaster))	x	Allstar	-	+	-	+	+ e
CPRO 90025	NL	c	Allstar	x	Korona	+	+	+	+	+ e
Darrow	USA	u	MDUS2713 (Redglow x Surecrop)	x	MDUS2787 ((Fairland x Midland) x (Midland x Md683))	+	+	+	+	+ a
Delite	USA	u	Albritton	x	MDUS2650 (((Blakemore x Md683) x Midland) x (Fairpeake x (Aberdeen x Redheart)))	+	-	+	-	+ a
Earliglow	USA	u	MDUS2359 (Fairland x Midland)	x	MDUS2713 (Redglow x Surecrop)	+	+	+	+	+ a
Guardian	USA	u	NC-1768*	x	Surecrop	+	+	+	+	+ a
Hood	USA	u	ORUS 2315	x	Puget Beauty	+	+	+	+	+ a
Linn	USA	u	MDUS3184 (NC-1768* x Surecrop)	x	ORUS 2414	-	-	+	+	+ a

Table 1 continued

Genotype name	Country of Origin[1]		Parental Plants ♀	♂	OPO 16C	OPC 8D	OPO 8A	SCAR $R1_a$	*Rpf1*[2]
Md683	USA	c	BK-46 (Frith selfed)	x Fairfax	+	+	+	+	+ a
MDUS3184	USA	u	NC-1768*	x Surecrop	+	+	+	+	+ b
Perle de Prague	UK	c	Unknown	x Unknown	+	-	-	-	+ a
Redchief	USA	u	same as MDUS3184		+	+	+	+	+ a
Scott	USA	u	Sunrise	x Tioga	+	+	+	-	+ f
Siltez	USA	u	ORUS 2012	x ORUS 1816	-	+	+	+	+ a
Stelemaster	USA	u	Fairland	x Md683	+	+	+	-	+ a
Sunrise	USA	u	US 4152 (Tennessee Shipper x Maytime)	x Stelemaster	+	+	+	-	+ a
Surecrop	USA	u	Fairland	x MDUS1972 (Blakemore x Md683)	-	+	+	+	+ f
Tribute	USA	u	EB 18 ((NC-1768* x Surecrop) x Cal 65.65-601)	x MDUS4258 ((Redglow x Surecrop) x (Midland x Sunrise))	+	-	+	-	+ f
Tristar	USA	u	same as Tribute		+	+	+	+	+ a
Yalova-4	TU	c	Cengelköy	x Aliso	-	-	+	+	+ e
Yalova-15	TU	c	Cengelköy	x Tiago	+	-	+	+	+ e, g

* NC-1768 complex cross: ((Fairpeake x (Aberdeen x Redheart)) x Tennessee Beauty)

1 Country indicates the breeding origin and the symbols indicate our source of the plant material:
- c: CPRO-DLO strawberry collection and breeding program, The Netherlands
- u: USDA National Clonal Germplasm Repository, Corvallis, OR, USA

2 *Rpf1* data from:
- a: Van de Weg (1997)
- b: Artificial inoculation with known races at Beltsville
- c: Van de Weg et al. (1997a)
- d: Tests on a naturally infested fields on which *Rpf1* is effective, Van de Weg et al. (1997b).
- e: Artificial inoculation with known races at CPRO-DLO
- f: Maas et al. (1989)
- g: resistance or susceptibility is based on the pedigree of the parents.

Table 2: Susceptible European and North American strawberry *rpf1* genotypes tested with three RAPD markers (OPC-8D, OPO-8A and OPO-16C) and the SCAR-$R1_a$.

Genotype name	Country of Origin[1]		Parental Plants ♀		♂	OPO 16C	OPC 8D	OPO 8A	SCAR $R1_a$	*Rpf1*[2]
52 AC 18	UK	c	Unknown	x	Unknown	-	-	-	-	- a
Aberdeen	UK	c	Unknown	x	Unknown	+	-	-	-	- a
Avanta	NL	c	(Induka x Sivetta)	x	(Karina x Precoce di Romagna)	+	-	-	-	- g, d
Blakemore	USA	c	Missionary	x	Howard 17	+	-	-	-	- a
Bogota	NL	c	Zb.53-11	x	Tago	+	-	-	-	- e
Brighton	USA	u	Tufts	x	Cal 65.65-601	+	-	-	-	- h
Cal 42.8-16	USA	u	(Sierra x (Blakemore x Nich Ohmer))	x	((Royal Sovereign x Howard 17) x (Royal Sovereign x Howard 17))	-	-	-	-	- h
Cambridge Favourite	UK	u	(Etterburgseedling x Avant Tout)	x	Blakemore	+	-	-	-	- i
Cambridge Vigour	UK	u	US 3378 (Aberdeen x Fairfax)	x	Early Cambridge	-	+	-	+	- a
Cavalier	CAN	u	Valentine (Howard 17 x Vanguard)	x	Sparkle	+	-	-	-	- g, j
Chandler	USA	u	Douglas	x	Cal 72.361-105	-	-	-	-	- e
Climax	UK	c	(TD-8 (CC-6 O.P) x (Frith O.P))	x	Aberdeen	+	-	-	-	- a
Columbia	USA	u	WSU 157	x	WSU 175	+	-	-	-	- b
CPRO 87018	NL	c	Elsanta	x	(Cambridge Favourite x (Sivetta x Precoce di Romagna))	+	-	-	-	- e
CPRO 89028	NL	c	((Tamella x Redgauntlet) x MDUS2700 (Pocohantas x Stelemaster))	x	Allstar	+	+	-	-	- e
CPRO 90013	NL	c	(Rapella x Cambridge Favourite)	x	Elsanta	+	-	-	-	- g/i/e/d
CPRO 90017	NL	c	(Rapella x Cambridge Favourite)	x	Gelria	+	+	-	-	- g/i/d
Del Norte	USA	c	*F. chiloensis* (random selection)	x	*F. chiloensis* (random selection)	-	-	-	-	- a
Douglas	USA	u	Tufts	x	Cal 64.57-108	-	-	-	-	- k
Elvira	NL	c	Gorella	x	Vola	+	-	-	-	- e
Fairfax	USA	u	Ettersburg 450 Royal Sovereign Howard Supreme	x x x	Howard 17 or Howard 17 or Ettersburg 450	+	-	-	-	- j

Table 2 continued

Genotype name	Country of Origin[1]		Parental Plants ♀		♂	OPO 16C	OPC 8D	OPO 8A	SCAR R1a	*Rpf1*[2]
Florida Belle	USA	u	Sequoia	x	Earlibelle	+	-	-	-	- b
Gorella	NL	c	Juspa	x	MDUS3763 (Suwannee x Midland)	-	-	-	-	- e
Grenadier	CAN	u	Valentine	x	Fairfax	+	-	-	-	- g
Holiday	USA	c	Raritan	x	NY-844 ((Redglow x Tennessee Shipper) x Redglow)	+	-	-	-	- g/a
Howard 17	USA	u	Crescent	x	Howard 1	+	-	-	-	- j
Jerseybelle	USA	u	NJ 953 ((Lupton x Aberdeen) x Fairfax)	x	NJ 925 (Pathfinder x Fairfax)	+	-	-	-	- l
Jucunda	?	c	Unknown	x	Unknown	-	-	-	-	- h
Karola	NL	c	(Gorella x Midway)	x	Karina	+	-	-	-	- e
Kent	CAN	c	Frogmore Late Pine	x	Raritan	+	-	-	-	- a
Lambada	NL	c	(Sivetta x Holiday)	x	(Karina x Primella)	+	-	-	-	- e
Lupton	USA	u	Joe	x	Gandy	-	-	-	-	- h
Lassen	USA	u	(Blakemore x (Marshall x Fendalcinno))	x	((Nich Ohmer x (Royal Sovereign x Howard 17)) x (Marshall x Fendalcinno))	-	-	-	-	- h
Macherauch's Frühernte	DL	c	Geneva	x	Deutsch Evern	+	-	-	-	- h
Marmolada	IT	c	Gorella	x	Unknown	-	-	-	-	- e
Marshall UCM 3585	USA	u	Unknown	x	Unknown	+	-	-	-	- h
Micmac	CAN	c	Tioga	x	K61-87 (Guardsman S_1)	-	-	-	-	- a
Midland	USA	u	Howard 17	x	Redheart	-	-	-	-	- j
Midway	USA	u	Dixieland	x	Temple	+	-	-	-	- m, f
Mrak	USA	c	Hecker	x	Aiko	+	-	-	-	- e
Redcoat	CAN	u	Sparkle	x	Valentine (Howard 17 x Vanguard)	+	-	-	-	- g/a
Redgauntlet	UK	c	NJ-1051	x	Climax	+	-	-	-	- a
Redglow	USA	u	Fairland	x	Tennessee Shipper	-	+	-	-	- g, b
Royal Sovereign	UK	u	Nobel	x	King of the Earlies	+	-	-	-	- h
Saladin	UK	c	Auchinicruive 61 AP 60	x	MDUS2650 (((Blakemore x Md683) x Midland) x (Fairpeake x (Aberdeen x Redheart)))	+	-	-	-	- a

Table 2 continued

Genotype name	Country of Origin[1]		Parental Plants ♀		♂	OPO 16C	OPC 8D	OPO 8A	SCAR $R1_a$	*Rpf1*[2]
Selva	USA	c	Cal 70.3-117	x	Cal 71.98-605	-	-	-	-	- e
Shucksan	USA	u	(Northwest x Sierra)	x	Columbia	+	-	-	-	- b
Senga Sengana	DL	c	Markee	x	Sieger	-	-	-	-	- a
Sierra	USA	u	Nich Ohmer	x	((Royal Sovereign x Howard 17) x (Marshall x Howard 17))	+	-	-	-	- h
Talisman	UK	c	NJ-1051	x	Climax	+	-	-	-	- e
Yaquina A	USA	c	*F. chiloensis* (random selection)	x	Unknown	-	-	-	-	- a
Yaquina B	USA	c	*F. chiloensis* (random selection)	x	Unknown	-	-	-	-	- a

1 see Table 1

2 *Rpf1* data from:

a-g: see Table 1

h: Darrow (1966), and/or bred during a time when genotypes were not selected for red stele resistance

i: Kennedy and Duncan (1988)

j: Stembridge (1961)

k: unpublished data of the USDA-ARS, Beltsville, MD

l: Tests on a naturally infested fields on which *Rpf1* is effective, Van De Weg & Meulenbroek (unpublished data)

m: Law and Milholland (1992)

Conservation of the markers linkage to *Rpf1*

The molecular and pedigree data show that OPO-8A and the SCAR marker have been conserved in many crosses over successive generations. This is illustrated by the pedigree of the breeding selection CPRO 90025, which possesses *Rpf1*, in which the linkage with these markers was conserved through five generations (Fig. 3).

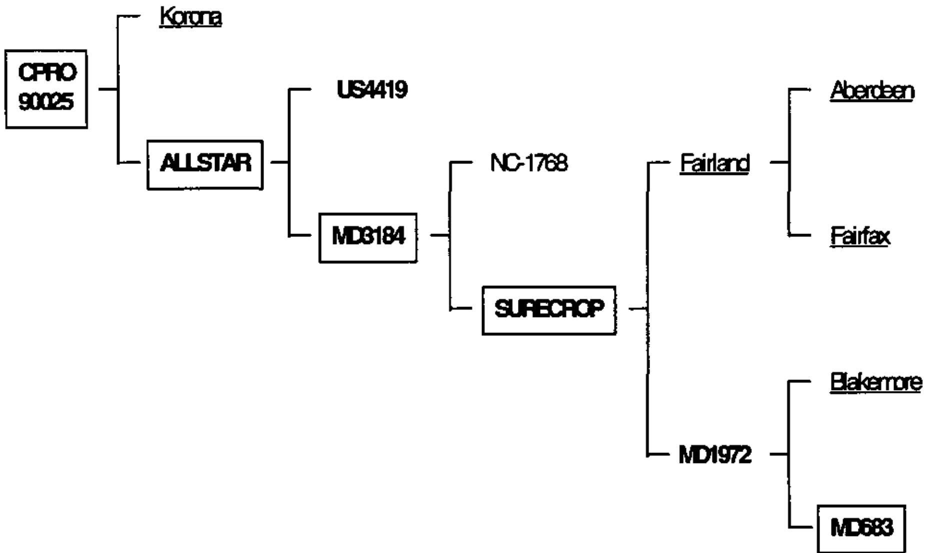

Figure 3. Pedigree of the strawberry selection CPRO 90025. *Rpf1* genotypes are in bold while *rpf1* genotypes are in normal text. Genotypes which possess both RAPD OPO-8A and SCAR-R1_a are indicated by a box while genotypes that lacked these markers are underlined. Genotypes that have neither a box nor an underline were not tested.

Prediction of *Rpf1* in genotypes

Seven genotypes were tested for which the presence or absence of *Rpf1* was unknown since the required data on resistance were lacking. For six of these the absence of *Rpf1* was predicted from the absence of both of the two flanking markers ('53 Q 13', 'Ettersburg 121', 'Mimek', 'Puget Beauty', 'Totem', and 'Tyee'). The seventh genotype, 'Olympus', could not be classified as being recombinant since it had OPO-8A but lacked SCAR-R1_a. An additional 40 susceptible genotypes, whose parents are *rpf1* susceptible and absent for the markers (OPO-8A and SCAR-R1_a), were assessed with the two nearest flanking markers. Each genotype screened gave the expected results (results not shown).

Discussion

The data show that the OPO-8A and the SCAR-R1$_a$ markers are highly conserved in the resistant genotypes and absent in the susceptible genotypes tested. The *Rpf1* genotypes assessed originated from breeding programmes in The Netherlands, United States, Canada, Scotland, and Turkey (Table 1). The linkage of the markers and the *Rpf1* gene was conserved in most of these genotypes irrespective of the place where they were bred. These markers can therefore be used for marker-assisted selection in breeding programmes, and pedigree-analysis.

Marker-assisted selection

The RAPD and SCAR markers can be used for marker-assisted selection aiming at the efficient introgression of *Rpf1* into, and the pyramiding of resistance genes in new cultivars, provided that not all genes are present in the same cluster. Red stele resistance tests are expensive and laborious, are affected by environmental factors, and suffer from incomplete resistance and epistatic effects among resistance genes. Additionally, in Europe *P. fragariae* is a quarantine pathogen requiring special laboratory facilities. In contrast, RAPD and SCAR makers are relatively inexpensive, reliable, and easily and quickly screened in that the marker is either present or absent, intermediate scores are not possible. The present markers may therefore encourage the breeding for red stele resistance since this becomes more economically and technically feasible.

The best prediction of a seedlings' resistance is by the use of two flanking markers on either side of the *Rpf1* gene, since this reduces the chance of false positives to a minimum when compared to the use of just a single marker. In this respect OPO-8A and the SCAR-R1$_a$ can be used due to their strong linkage to *Rpf1*. In some genotypes, like 'Stelemaster' and its descendants, RAPD OPO-16C is linked to *Rpf1* instead of SCAR-R1$_a$. OPO-8A and OPO-16C could then be used as the flanking markers. However, the latter is only applicable if OPO-16C is lacking in the other parent.

RAPD OPO-8A was used since no SCAR is currently available for this marker. Its conversion into a SCAR has been hampered due to the marker's high molecular weight (1650 bp) and its close presence to an intense band (Fig. 2) (Haymes et al. 1997b).

OPC-8D produces an intense polymorphic marker of 510 bp that can easily be assessed for presence or absence (Fig. 1) (Haymes et al. 1997a). Since OPO-8A is not always easy to rate, due to being close to

another intense band, OPC-8D can be used for preselection. This useful approach is suitable for crosses in which OPC-8D is only present as being linked to *Rpf1*. When crossing-over does not occur between OPC-8D and the SCAR, in which case both markers are being either present or absent, then OPC-8D can be used to determine resistance (e.g. 'Cornwallis'). If crossing-over occurred then additional tests are required using OPO-8A.

Simultaneously with SCAR-$R1_a$ a second set of SCARs, SCAR-$R1_b$, was developed from the same DNA sequence (Haymes et al. 1997b). This SCAR was also tested throughout the research and gave identical results to that of SCAR-$R1_a$.

Of the seven additional genotypes tested for which the presence of *Rpf1* was not previously known 'Olympus' may have obtained the gene though its ancestry. The molecular data are not conclusive since one of the flanking markers (SCAR-$R1_a$) was absent. If the crossing-over occurred between *Rpf1* and SCAR-$R1_a$ then 'Olympus' should possess the gene, however if the crossing-over event occurred between *Rpf1* and OPO-8A then most likely the gene was lost.

The reasons for the absence of both OPO-8A and SCAR-$R1_a$ and the presence of OPO-16C in 'Perle de Prague' could not be determined since its parentage is unknown. One possible explanation is that two separate crossing-over events occurred among these flanking markers and the *Rpf1* gene due to which *Rpf1* became linked to OPO-16C instead of the SCAR. Another possibility is that 'Perle de Prague' has a different resistance gene which has not yet been determined in the strawberry-*P. fragariae* gene-for-gene model, similar to *Rpf1* in disease response for the isolates tested.

Identification of genotypes homozygous for *Rpf1*

The ability to select homozygous *Rpf1* genotypes would be a strong asset in many breeding programmes. Dominant (molecular) markers are generally not suitable to distinguish between heterozygous and homozygous genotypes. However, the present markers allow such a discernment in two types of R x R progenies. The first is where *Rpf1* is linked to OPO-16C in one parent (e.g. 'Scott') and in the other parent the gene is linked to SCAR-$R1_a$ in the absence of OPO-16C (e.g. Yalova-4). These markers are alternate alleles of the same locus which segregates disomically (Haymes et al. 1997a). Consequently, *Rpf1*-homozygous progeny would have both OPO-16C and the SCAR, heterozygous progeny would have one of the markers, while *rpf1* homozygous progeny would lack both. The second type of R x R cross requires *Rpf1* in both parental genotypes to be linked to SCAR-$R1_a$ as

well as the presence of OPO-16C in the homologous chromosome (e.g. 'Cornwallis', Md683, 'Tristar', and 'Yalova-15'). *Rpf1*-homozygous progeny would lack the OPO-16C marker and have the SCAR marker, *Rpf1*-heterozygous would have both the SCAR and OPO-16C marker, while *rpf1* homozygous would lack the SCAR and have OPO-16C. For example, this is the case in the selfed progeny of Md683, in which seven out of 24 are predicted to be homozygous resistant, 15 to be heterozygous, and two to be homozygous susceptible for *Rpf1* according to the molecular data (results not shown). These numbers fit to the expected 1:2:1 segregation ratio at the 95% level ($\chi_2{}^2 = 3.9$).

Pedigree-analysis: presence of *Rpf1* in parentages

The occurrence of *Rpf1* in many Eastern North American breeding selections and in some selections from the west coast of the United States (e.g. Oregon: 'Benton', 'Linn', and 'Hood'), Scotland, and The Netherlands, was not surprising since they all have the oldest known source of *Rpf1* resistance ('Frith') somewhere in their ancestry. These *Rpf1* selections were predominantly derived from Md683, a second generation descendent of 'Frith' (Reid 1952; Scott et al. 1984). Interestingly, *Rpf1* is also present in the Oregon cultivar 'Siletz' and the Turkish cultivars 'Yalova-4' and 'Yalova-15' although to the knowledge of the authors 'Frith' is not in their ancestry. The probability of the same resistance gene arising independently is highly unlikely and therefore we believe that this *Rpf* gene came from a much older common ancestral genotype and that the linkage of the markers was conserved even through these earlier generations.

'Tribute' and 'Tristar' have two possible genitors of *Rpf1*, 'Surecrop' and 'Sunrise' (Table 1). Surecrop's *Rpf1* is linked to the SCAR while in 'Sunrise' *Rpf1* is linked to OPO-16C. Since in 'Tribute' the gene is linked to the OPO-16C and not to the SCAR, the *Rpf1* allele should have come from 'Sunrise'. In contrast, the SCAR-R1_a of 'Tristar' should be due to the *Rpf1* allele received from 'Surecrop'. The presence of OPO-16C marker in 'Tribute' and 'Tristar' (both released in 1981) can be traced back to 'Fairfax', a cultivar released in 1933.

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Chapter 6

Agrobacterium-mediated transformation of 'Alpine' *Fragaria vesca*, and transmission of transgenes to R1 progeny.

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Abstract

Agrobacterium-mediated transformation was used to stably introduce the GUS and NPT-II marker genes into 'Alpine' *Fragaria vesca* accession FRA 197, a diploid (2N=2X=14) strawberry. Primary (R0 generation) transformants derived from a single clump of kanamycin-resistant callus were vegetatively propagated to produce five R0 runner plants. The presence of the GUS and NPT-II genes in all R0 runner plants was confirmed by PCR. Southern analysis detected the presence of two sites of NPT-II insertion into genomic DNA of the R0 transformants, and indicated that all five R0 runner plants were derived from a common transformation event. When R1 generation seedlings obtained via self-pollination of the R0 runner plants were tested by histochemical assay for GUS expression, 591 were GUS positive and 39 were GUS negative. The segregation data fit a 15:1 ratio (0.5>p>0.25), indicating the independent segregation of two transgene insertion loci. These results demonstrate the suitability of 'Alpine' *F.vesca* for transgene research in strawberry.

Key words: PCR, progeny-analysis, strawberry

Introduction

The development of in vitro regeneration and genetic transformation systems for the cultivated strawberry, *Fragaria* x *ananassa*, has opened up the opportunity for strawberry improvement through genetic engineering (Nehra et al. 1992). Marker genes have been introduced into the cultivated strawberry, *F.* x *ananassa* (*Fragaria* species) by *Agrobacterium*-mediated transformation (James et al. 1990; Nehra et al. 1990a, 1990b), and by electroporation of protoplasts (Nyman and Wallin 1992). A gene of economic interest, the S-adenosylmethionine hydrolase (SAMase) gene for control of ethylene biosynthesis, was introduced into *F.* x *ananassa* via an *Agrobacterium* system (Mathews et al. 1995). Of the foregoing citations, only James et al. (1990) reported the sexual transmission of transgenes to R1 generation progeny.

As an octoploid, *F.* x *ananassa* (2n=8x=56) is a difficult subject for genetic investigation. The genomic complexity of the cultivated strawberry may pose difficulties for the analysis of transgene expression and transmission. As an alternative system for many aspects of strawberry genetic research, *F. vesca* (2n=2x=14), offers several advantages over its octoploid relative (Brown and Wareing 1965; Ahmadi et al. 1988). In addition to the obvious advantage of a diploid genome structure, the so-called 'Alpine' forms of *F. vesca* are naturally self-pollinating and can be highly inbred without loss of fertility. Several useful *F. vesca* mutants have been described (Galletta and Maas 1990; Davis and Pollard 1991), and an *F. vesca* genetic linkage map has been constructed (Davis and Yu 1997).

The susceptibility of *F. vesca* to *Agrobacterium*-mediated transfer of marker genes has been demonstrated (Haymes and Davis 1993; Mansouri et al. 1996). Mansouri et al (1996) used seeds collected from wild *F. vesca* populations to establish in vitro plantlets as explant sources. Transgenic plants were regenerated, but the production of R1 progeny was not reported. A potential disadvantage of wild *F. vesca* for use in transformation and other genetic studies is that it is photoperiod sensitive, flowering only under short day conditions (Brown and Wareing 1965; Ahmadi et al. 1990). In contrast, *F. vesca* 'Alpine' forms are day neutral, and will flower and set fruit continuously in a greenhouse under ambient light (Brown and Wareing 1965; Ahmadi et al. 1990). In the present report, we describe the *Agrobacterium*-mediated transformation of an 'Alpine' *F. vesca*, and segregation analysis of the transgenes in R1 progeny.

Material and methods

Plant material and culture

F. vesca accession FRA 197 was obtained as a runner plant from the USDA National Clonal Germplasm Repository, Corvallis, Oregon, USA, and was runner-propagated and maintained in a greenhouse. FRA 197 is day neutral in its flowering habit and is highly self-fertile.

Seeds resulting from natural self-fertilization of FRA 197 plants were used to establish in vitro plantlets as explant sources. Seeds were wrapped in cheesecloth and soaked in an aqueous soap solution (1:100 v/v of Ivory liquid detergent) for 10 min., then gently agitated (40 rpm) for 10 min. in 30% (v/v) commercial bleach (Clorox: 5.25% (v/v) sodium hypochlorite). Seeds were rinsed three times in sterile distilled water and placed on basal medium (ph 5.6) consisting of MS salts, B5 vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar (Gibco Phytagar). All cultures were incubated in a growth chamber at 25°C under 12 h/day photoperiod (12.5 $mE.m^{-2}s^{-1}$ light intensity) unless stated otherwise. After seed germination and appearance of the first true leaf, seedlings were transferred to Magenta GA7 jars (Magenta Corp., Chicago, IL) containing 50 ml of basal medium. After 6 or more weeks of culture in GA7 jars, plantlets were used as explant sources for transformation experiments.

Sensitivity to kanamycin

Kanamycin sensitivity of leaf disks and petiole sections was assessed prior to *Agrobacterium* transformation to determine the concentration of kanamycin needed for effective selection of transgenic plants. Twenty leaf segments (approximately 1 cm^2) and 20 petiole sections (approximately 2-3 mm in length) per plate were placed onto 50 ml plates of basal medium containing a kanamycin concentration of either 0, 10, 25, 50, 75 or 100 mg/l. Two replica plates were used for each kanamycin level. After 4 weeks of culture, explants were evaluated in terms of general appearance and color, and presence/absence of callus or shoots.

Transformation of plant material

The binary vector plasmid pBI121 carrying both CaMV 35S::GUS (β-glucuronidase) and Nos::NPT-II (neomycinphosphotransferase-II) (marker genes (Jefferson et al. 1987) was mobilized from *E. coli* strain HB101 into *A. tumefaciens* strain LBA4404 by triparental mating (Ditta et al. 1980).

Explants were co-cultivated with *A. tumefaciens* strain LBA4404 using a modification of the protocol of Nehra et al. (1990b). Agrobacteria were suspended to a final cell density of 106-108 cells/ml in an aqueous co-cultivation solution containing 0.85% NaCl and 50 uM acetosyringone (Aldrich Chemical Co., St. Louis, MO). Twenty leaf segments (approximately 1 cm^2) and 20 petiole sections (approximately 2-3 mm in length) excised from 8-week-old plantlets were co-cultivated with 15 ml of the bacterial suspension under gentle agitation (40 rpm) for 20 min. at room temperature. Control leaf disks and petioles were similarly treated in a co-cultivation solution lacking bacteria.

Subsequently, tissue sections were rinsed three times with sterile distilled water, blotted dry on sterilized filter paper and placed on shoot induction medium (SIM) consisting of basal medium plus 10 mM BA (6-benzyladenine), 1 mM NAA (naphthaleneacetic acid) and 250 mg/l mefoxin (a bacterial static from Merke, Sharp and Dohme Inc., West Point, PA). After five days, leaf and petiole sections were transferred to SIM supplemented with 50 mg/l kanamycin (SIM + 50K), and subcultured every six weeks to new SIM + 50K. Beginning with the third subculture, the kanamycin concentration was reduced to 25 mg/l (SIM + 25K). Shoots that proliferated on the SIM + 25K were transferred to basal medium (hormone free) containing 25 mg/l kanamycin and 250 mg/l mefoxin.

Putative transgenic plants (R0 generation) were transferred to a greenhouse and were physically isolated from all other strawberry plants. Several runner plants were propagated from the initial R0 plants in an effort to eliminate chimerism. These R0 runner plants were utilized in all subsequent experiments. Control FRA 197 and R0 runner plants were allowed to self fertilize, and seeds were harvested separately from each plant.

Molecular analysis of putative transformants

DNA isolated from R0 runner plants and control plants using the method of Haymes et al. (1997) was tested for the presence of the NPT-II and GUS marker genes by the polymerase chain reaction (PCR). GUS and NPT-II PCR primer pairs were designed using the computer program 'Primer' of Lincoln et al. (1991). The NPT-II primer sequences (5'-3') were TCCAGATCATCCTGATCGACAAG and CAAGATGGATTCCACGCA GGTTC, based on the known NPT-II sequence (Beck et al. 1982). The GUS primer sequences (5'-3') were CAACGCTGACATCAC and ACTGGCAGACTATCC, based on the known GUS sequence (Guerrero et

al. 1990). The predicted sizes of the NPT-II and GUS amplification products are 503 bp and 230 bp, respectively.

Each 25 μl PCR reaction mixture contained 0.5 units *Taq* polymerase and associated buffer (Life Technology, Gaithersburg, MD), 3.5 mM MgCl2, 0.1 mM each dNTP, 0.4 M each of two primers (NPT-II or GUS), and 50 ng plant DNA or 20 ng plasmid DNA. A Perkin Elmer Cetus Thermal Cycler was used for the PCR using a step cycle program (94°C 50 sec, 60°C 1 min. and 72°C 2 min. for 35 cycles) linked to a final 72°C 7 min. extension.

For Southern blot analysis, 10 μg of total genomic DNAs from transgenic R0 runner plants and control plants were digested with *Hind*III (Promega Corp., Madison, WI). The digested DNA was electrophoresed on a 1.0% (TAE) agarose gel and vacuum transferred to a nylon membrane (Hybond-N+, Amersham). The membrane was utilized for Southern blot analysis according to manufacturer's instructions. The Amersham Megaprimer DNA labeling kit was used to synthesize ^{32}P-labeled NPT-II probe from the 1.8 kb NPT-II insert released by digesting the Tn5-containing plasmid pACK1 (Mylnarova et al. 1994) with *Hind*III and *Bam*HI.

Histochemical analysis of R0 and R1 plants

Seeds collected from R0 runner plants and untransformed control plants were aseptically germinated to obtain R1 seedlings, which were subjected to a GUS activity assay in two groups, as described below. Surface sterilized seeds were transferred in batches of 25-50 into petri dishes containing either water-soaked filter paper (Whatman #1) or basal medium, and incubated for two weeks to allow germination. Root sections or entire seedlings were incubated in the wells of a 96 well microtiter plate (Costar Corporation, Cambridge, MA) in 100 μl X-gluc substrate solution (Jefferson et al. 1987) per well. The microtiter plates were placed under vacuum for 5 min. to ensure the penetration of the substrate solution into the tissues, then incubated overnight at 37°C in the dark. After incubation in the X-gluc, GUS activity was indicated by blue coloration of the tissues. Leaf sections excised from R0 runner plants were also tested for GUS activity.

A first group (set 1) of 414 R1 seedlings and 200 control seedlings were assessed only for GUS activity. After the emergence of the first true leaf, the root tip was removed from each seedling and assayed for GUS activity as described above. Following incubation in the X-gluc solution, root color was observed directly in the Microtiter plates. The shoots of

these seedlings were to have been used in a kanamycin resistance assay, but were lost due to subsequent contamination.

The second group (set 2) of 216 R1 seedlings and 50 control seedlings were tested for kanamycin resistance prior to the GUS assay. Seeds were germinated on basal medium, then seedlings were transferred to SIM + K when the first true leaves emerged. After 4 weeks, sensitivity (+/-) of each seedling to kanamycin was rated based on color and general appearance. Each seedling was then tested for GUS Activity by histochemical analysis. Following incubation in the X-gluc solution, seedlings were removed from the microtiter plates, then boiled for 5 min. in 70% ethanol to remove chlorophyll to allow easier recognition of blue color in the shoots.

Results and Discussion

Kanamycin sensitivity of explant material.

Callus formation occurred on antibiotic-free control plates, and to a lesser extent on plates with 10 mg/l kanamycin concentration. A 25 mg/l kanamycin concentration caused chlorosis, and eventual necrosis in all explants by the end of the fourth week. Kanamycin concentrations of 50 mg/l and higher caused all explants to become necrotic by the third week. In all previous reports of *Agrobacterium*-mediated transformation of strawberry, kanamycin concentrations of 25-50 mg/l were used in the initial selection medium (James et al. 1990; Nehra et al. 1990a, 1990b; Mathews et al. 1995; Mansouri et al. 1996). Accordingly, we chose a kanamycin concentration of 50 mg/l for the initial selection of transformants.

Transformation and selection.

During the initial five days on non-selective SIM medium, all co-cultivated and control explant material retained a healthy green color. After transfer to SIM + 50K (selective medium), uninoculated control explants and most of the co-cultivated explants became completely necrotic within 3-4 weeks. In contrast, control leaf and petiole sections maintained on non-selective SIM exhibited shoot regeneration by the end of the fourth week in culture. Shoots formed around the periphery of control leaf disks directly, and callus formation occurred at the ends of the petiole sections, with shoots developing from the callus tissue.

Two of the co-cultivated petiole sections retained a healthy green color after transfer to SIM + 50K, and showed evidence of swelling after a

few weeks. Four of the 20 leaf explants also remained green in color but never exhibited swelling or callus production. The kanamycin concentration was lowered to 25 mg/l at the third subculture to reduce antibiotic stress on the explants. Soon after transfer to SIM + 25K, the two swelling petiole sections began to produce callus. After 4 weeks on SIM + 25K, callus growth ceased on one of the petiole sections, but the other continued to produce callus prolifically. This callus tissue was then separated from the petiole section and allowed to proliferate on SIM + 25K, where it gave rise to multiple shoots, and then whole plantlets when transferred to hormone-free basal medium. These R0 plantlets were removed from culture and, after a period of radual acclimation, were established in pots in an isolated greenhouse. In an effort to eliminate chimerism in the R0 plants, the R0 plants were propagated by means of runners to produce five R0 runner plants: TR1, TR2, TR3, TR4 and TR5.

Molecular characterization of transgenic runner plants

In the histochemical assay, leaf segments taken from R0 runner plants tested positive for GUS expression, while tissues from untransformed FRA 197 plants tested negative. These results, combined with the fact that the primary R0 plants were derived from callus that had survived kanamycin selection, indicated that functional copies of the GUS and NPT-II genes were present in the transformants.

When the R0 runner plants and FRA 197 untransformed control plants were tested for presence of the NPT-II and GUS genes by PCR assay, the expected ~503 bp NPT-II and ~230 bp GUS bands were found in the transformants and were absent in the untransformed controls (Figure 1). Results for R0 runner plant TR5 (not shown) were the same as those for R0 runner plants TR1-TR4 (Figure 1). As a positive control, the GUS and NPT-II products were also amplified from the pBI121 plasmid (Figure 1).

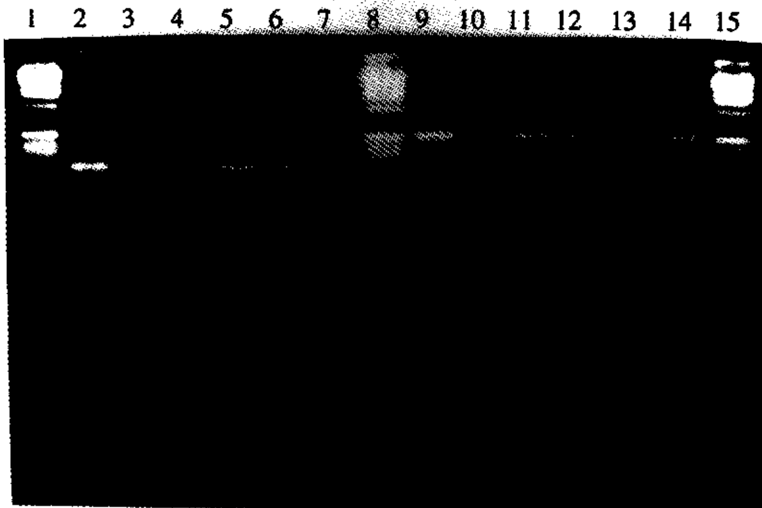

Figure 1: PCR amplification of control and transgenic runner plant (TR1-TR4) DNAs with GUS and NPT-II primers. Ln 1,8,15: 1 kb ladder. Ln 2: pBI121 (GUS). Ln 3: untransformed FRA 197 (GUS). Ln 4-7: R0 runner plants TR1, TR2, TR3, TR4, (GUS). Ln 9: pBI121 (NPT-II). Ln 10: untransformed FRA 197 (NPT-II). Ln 11-14: TR1, TR2, TR3, TR4 (NPT-II). Expected sizes of amplified PCR fragments were GUS (230 bp) and NPT-II (503 bp).

The insertion of the NPT-II marker gene into transformed plant genomic DNA was demonstrated by the results of Southern analysis (Figure 2). DNA from transgenic and control plants was digested with *Hind*III, which cuts at only one site - located between the GUS and NPT-II genes - in the T-DNA insert of pBI121. The NPT-II probe detected two restriction fragments of sizes 6.2 and 3.5 kb in the R0 transformant DNAs (Figure 2, lanes 3-6). Results from R0 runner plant TR-4 (not shown) were the same as for other R0 four plants (Figure 2). The probe did not hybridize to untransformed FRA 197 genomic DNA (Figure 2, lane 2) used as a negative control, but did hybridize to the 1.8 kb NPT-II insert from plasmid pACK1 used as a positive control (Figure 2, lane 1). All five R0 runner plants having the same two-banded restriction pattern suggests that the T-DNA integration sites were the same in all five plants, as would be expected if all five R0 plants were derived from a single transformation event. This interpretation is consistent with the fact that all of the primary R0 regenerants originated from the same clump of callus.

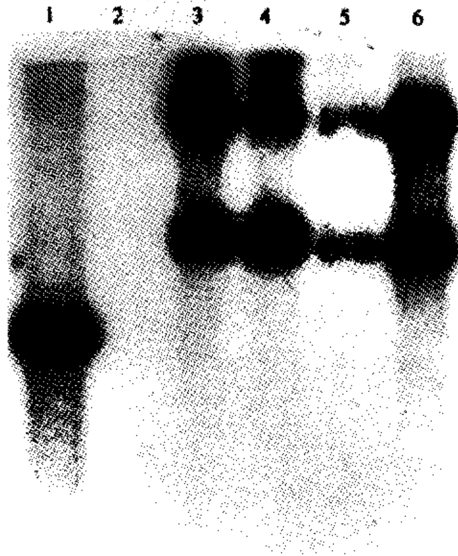

Figure 2: Southern blot of *Hin*DIII digested genomic DNA from R0 runner plants and an untransformed control plant, using an NPT-II probe. Ln 1: 1.8 kb NPT-II fragment obtained from *Hin*DIII and *Bam*HI digest of pACK1 (positive control). Ln 2: untransformed FRA 197. Ln 3-6: R0 runner plants TR1, TR2, TR3, and TR5, respectively. Band sizes were estimated by comparison to a moleuclar weight ladder (not shown).

The detection of two restriction fragments carrying NPT-II sequences in transformant DNA indicates that either i) there were two T-DNA insertion sites in the transformed genomes or that, ii) there were two T-DNA inserts located in tandem at a single site. The hypothesis of two independent insertion events was supported by the results of progeny tests, as described below.

Transmission of GUS and NPT-II genes to R1 progeny

The histochemical assay detected GUS activity in most of the R1 seedlings, showing that one or more functional GUS genes were passed from R0 plants to the segregating R1 progeny. The 200 control plant seedlings did not have any intrinsic GUS activity. Assayed control seedlings were white, while putative transgenic seedlings were completely blue (Figure 3). The ratio of GUS+:GUS- R1 seedlings was 390:24 in the first set of seedlings, and 201:15 in the second set (Table 1). Results were tabulated separately for R1 seedlings derived from R0 runner plants TR1-TR5: however, a Chi-square homogeneity test showed that the results from the five R1 families were homogeneous and could be pooled (Table 1).

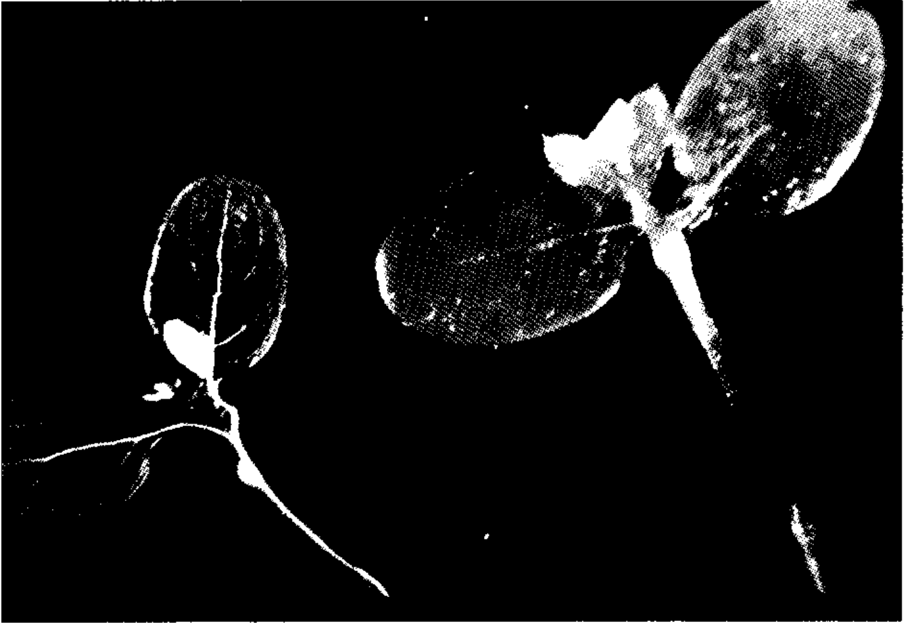

Figure 3: GUS histochemical assay of transformed R1 (right) and untransformed control (left) seedlings. Blue pigmentation indicative of GUS activity is present throughout entire R1 seedling. No intrinsic GUS activity is evident in the non- transformed plant.

The overall GUS+:GUS- ratio of 591:39 differed significantly (χ^2=118.88, $p<0.01$) from the 3:1 ratio expected for segregation of a single dominant gene, but fit the 15:1 ratio expected for independent segregation of two dominant GUS genes (Table 1). The segregation ratios within each of the five R1 progeny families also fit a 15:1 ratio. These results indicate that there were two independent sites of GUS transgene insertion in the genomes of the R0 plants.

Table 1. Segregation of GUS positive (+) versus GUS negative (-) transgene expression in R1 progeny of five transgenic (RO) runner plants. A χ^2 test is applied to the pooled data, using a 15:1 (GUS+:GUS-) expected ratio. TR3 or TR5 progeny were not included in sets 1 and 2, respectively.

RO Plant	Set 1 (+)(-)	Set 2 (+)(-)	Pooled (+)(-)	χ^2 (15:1)	P
	----- Number of R1 plants -----				
TR1	85 11	46 3	131 14	2.87	0.10>P>0.05
TR2	162 8	32 2	194 10	0.63	0.50>P>0.25
TR3	- -	63 5	63 5	0.14	0.75>P>0.50
TR4	70 1	60 5	130 6	0.78	0.90>P>0.75
TR5	73 4	- -	73 4	0.15	0.50>P>0.250
Sum χ^2				4.57	0.75>P>0.50
Total	390 24	201 15	591 39	0.01	0.50>P>0.25
Homogeneity test χ^2 (df=4)				4.56	0.50>P>0.25

Of the 216 R1 seedlings (set 2) tested for kanamycin resistance prior to the GUS assay, 201 were classified as resistant and 15 as susceptible. Of the 201 resistance seedlings, 199 were GUS+ and 2 were GUS-. Similarly, of the 15 susceptible plants, 13 were GUS- and 2 were GUS+. Thus, the presence or absence of kanamycin resistance was co-inherited with presence or absence, respectively, of GUS activity in all but 4 of the 216 R1 progeny tested for both features. The four deviating seedlings may have been misclassified in the NPT-II scoring, or one of the transgenes may have been mutationally inactivated or silenced in these four progeny plants. In any case, these results showed a predominant pattern of co-transmission of the GUS and NPT-II genes to the R1 progeny, as was expected given that the two genes were carried on the same T-DNA construct.

The results presented here provide the first demonstration of sexual transmission of transgenes in the diploid strawberry, *F. vesca*, and only the second such demonstration in any strawberry species. Previously, James et al. (1990) observed a 3:1 segregation ratio, indicative of a single site of transgene insertion, among 78 R1 progeny of transgenic octoploid (*F.* x *ananassa*) strawberry plants. The transmission genetic analysis performed by James et al. (1990) was hampered by the very slow and low germination rate (15% germination after several months in culture) of the R1 seeds. In

contrast, the diploid R1 seed obtained in the present study had a high (95-100%) rate of germination *in vitro* and in soil.

Although the success of the present study was based upon a single transformantion event, the results extend the development of genetic transformation systems for strawberry by demonstrating the suitability and usefulness of *F. vesca* 'Alpine' strawberries for conducting transgene research in *Fragaria*. R1 seeds were readily obtainable because of the self-fertility and day neutral flowering habit of the 'Alpine' accession, FRA 197, used in this study. Of the available 'Alpine' germplasm, FRA 197 is particularly well-suited for transformation studies because the R0 plants can be easily runner-propagated. In contrast, many 'Alpine' accessions, such as 'Baron Solemacher' and 'Yellow Wonder', are also non-runnering (Yu and Davis 1995).

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General Discussion

F. x *ananassa* is an autoallopolyploid species (2n=8x=56) of which the genetics of most traits is unknown and are likely to be complex due to its genetic constitution. Diploid and octoploid strawberries have previously been reported to cross-hybridise and to exhibit chromosome pairing during meiosis. This indicates homology between the genomes of the strawberry. As more genes are mapped in strawberries, conservation of markers in linkage groups among the various species should be assessed. Simplex variation for traits such as resistance to *Phytophthora fragariae*, *Verticillium*, *Collectotrichum*, and traits like fruit colour and daylength-sensitivity occurs naturally in all strawberries species. The diploid species is likely to be more suitable for genetic analysis of complex traits due to the lower genome number. For this reason, a RAPD map of the diploid strawberry was generated (Chapter 2).

Genome mapping in the diploid strawberry

The small genome size and the ability to grow the plants from seed quickly, make the diploid (2n=2x=14) *Fragaria vesca* a suitable species for mapping purposes. The F_1 behaved as a testcross in that one parent (FRA 364, a wild accession obtained from USDA National Clonal Germplasm Repository, Corvallis, OR, USA) was heterozygous and the other parent ('Yellow Wonder', an USDA cultivar) was homozygous, and recessive with respect to the evaluated RAPD markers. Therefore, a 1:1 segregation ratio was expected for both the dominant RAPD and codominant isozyme markers in the F_1 population but some markers deviated from this ratio. Some researchers discard molecular markers that segregate significantly differently from the expected ratio (Reiter et al. 1992) however, the deviating markers were shown to be linked to at least one other marker, except for four, therefore they were included in the mapping study. The F_1 population of 55 plants was determined to be large enough to accurately detect linkages less than or equal to a recombination frequency of 25%, according to the method of Hanson (1959). The 70 RAPD, 2 isozyme and one morphological marker were compiled into 5 distinct linkage groups using the JoinMap 2.0™ program (Chapter 2). It is possible due to chromosomal inversion, that two linkage groups could not be detected in this cross due to meiotic imbalance caused by having two different genomes (*F. vesca* and *F. viridis*) in the male parent. In a latter study using a different cross all seven linkage groups were mapped (Davis and Hu 1997).

Continuing research will be required to complete and saturate the diploid strawberry linkage map. A *F. vesca* linkage map will have various uses such as to determine the evolutionary homology between the diploid versus the octoploid strawberry genomes, allow direct identification of collinear chromosomes or between strawberry and other members of the Rosaceae family.

Mapping in outbreeding and polyploid species

Most procedures for linkage mapping use inbred (fully homozygous genotypes) as parents. When outbreeding species (heterozygous parents) like strawberry, alfalfa, apple, or potato are used then the original data sets must be adjusted to use existing procedures. In some designs the segregation of alleles from one parent only is used thus treating the F_1 population as a backcross population (see Chapter 2). This was the situation in the development of the RAPD map in the diploid strawberry.

Wu et al. (1992) described a method of mapping in polysomic polyploid species using molecular markers that could follow the distinct chromosomes provided that they have an unique allele fragment called a single-dose restriction fragment (SDRF). These SDRF can be used for linkage studies, thereby enabling the construction of a linkage map and ultimately localisation of interesting traits in polysomic polyploid organisms.

Chromosome mapping of such polyploids has not yet been possible when the individual chromosomes cannot be distinguished (e.g. strawberry). However, as in the strawberry, mapping is possible of traits that are shown to segregate in diploid fashion (see Chapter 3). In these situations complete linkage maps are not constructed but condensed (fine) maps around the gene of interest.

Identification of the *Rpf1* resistance gene region

One of the initial steps in gene mapping is to develop a population where the trait of interest can be easily identified. A F_1 monogenic segregating population of the commercial strawberry (*Fragaria* x *ananassa*) was used to map the *Rpf1* gene. The population, developed by colleague Van de Weg at CPRO-DLO,was derived from a cross between Md683 (*Rpf1* resistant) x Senga Sengana (*rpf1* susceptible), and behaved as a diploid for mapping purposes of *Rpf1*. Md683 is expected to be resistant to at least 16 races of *P. fragariae*, including the American (A): A1, A3, A4, A6, A9, A10 and Nova Scotia (NS): NS2 isolates (Van de Weg 1997), while Senga Sengana is susceptible to all races tested. Haymes et al. (1997) (Chapter 3)

previously confirmed that the *Rpf1* gene is a single dominant inherited gene by the segregation of the F_1 mapping population to the seven RAPD markers linked to the gene.

High density molecular mapping

The precise localisation of a gene requires many markers saturating the chromosomal region comprising the trait. High density molecular linkage maps of the entire genome can be obtained by using molecular markers such as RAPD, RFLP, STS or AFLPs. Additional markers can also be identified for only the small region of the genome in which the trait of interest is located. These markers can be obtained most efficiently by genotypes having the trait, and by pooling DNA by Bulked Segregant Analysis (BSA). This is done based on prior genotypic information and subsequently performing RAPD (see Chapter 3) and/or AFLP analysis on these pooled samples.

Fine maps of regions around resistance genes have been made in a variety of species such as stem rust and leaf rust resistance in barley (Borokova et al. 1995; Poulsen et al. 1995), anthracnose and rust resistance in common bean (Miklas et al. 1993; Adam-Blondon et al. 1994; Johnson et al. 1995), white rust (*Alburgo candida*) resistance in *Brassica napus* (Ferreira et al. 1995), black leaf spot resistance in Chinese elm (Benet et al. 1995), *Verticillium* resistance in lettuce (Michelmore et al. 1991), powdery mildew resistance in pea (Timmerman et al. 1994), potato leaf roll virus and potato cyst nematode resistance in potato (Barker et al. 1994; Pineda et al. 1993), gall midge resistance in rice (Nair et al. 1995), powdery mildew resistance genes in tomato (Chunwongse et al. 1994; Van der Beek et al. 1994), and leaf rust resistance in wheat (Schachermayr et al. 1995).

With the technique of BSA a concentrated map of the *P. fragariae* resistance gene *Rpf1* was constructed using RAPD markers (Chapter 3). This map was made possible due to the monogenic segregation for this trait (Van de Weg 1997) and treating the F_1 progeny as a backcross population. The resistance gene was mapped with tightly linked flanking markers at 1.7 to 3.0 cM from the gene. Its location within the genome is still unknown due to the lack of a cytological or a complete molecular map of the octoploid strawberry. Therefore only two possibilities to eventually clone this gene remain open; map-based cloning and transposon tagging.

Control of *Phytophthora fragariae* by breeding using marker-assisted selection

P. fragariae resistance breeding is one of the primary targets of many breeding programmes, since the disease causes substantial damage without proper control mechanisms. *P. fragariae* can be controlled by a combination of levelling, good drainage, raised beds, and careful water control assisted by appropriate preplant soil fumigation. However, consumer and environmental concerns of chemical treatments are causing breeders to develop alternative means of controlling diseases and pests. Resistance tests for *P. fragariae* are labour intensive and expensive, therefore indirect selection by means of molecular markers is one method that is currently under investigation at CPRO-DLO.

Young and Tanksley (1989) demonstrated that marker-assisted selection during backcross programs could largely reduce both the number of required steps and the length of introgressed fragments. Marker-assisted selection can be employed to examine whether or not introgression of a desired trait has happened when molecular markers surround the gene. This procedure appears to be faster and easier than performing complicated tests to establish the phenotypic value of a plant . However, tight linkage of markers and trait is required to diminish the chance of recombination. A sequence characterised amplified region (SCAR) marker was designed based on one of the RAPD markers lined to *Rpf1* at 3.0 cM (Chapter 4). The developed dominant SCARs amplified DNA only from the resistant plants in the mapping population.

The DNA isolation mini-prep procedure (Chapter 5) in conjunction with marker-assisted selection was utilised to select recombinant plants in the screening of 932 strawberry plants. Seedlings were grown in a four meter area of the greenhouse and leaf material was collected when the plants were only 5 cm in height. Selection of the recombinant plants was done prior to any testing against the fungus. After the DNA was isolated, PCR amplification was performed in a microtiter plate according to Haymes et al. (1997) (Chapter 3), using three RAPD primers tightly linked and surrounding the resistance gene. The SCAR markers (Chapter 4) were later tested on the selected putative recombinants. Each primer was used a minimum of two times to guarantee that the observed polymorphism was correct. Due to the map location of the RAPD primers to the *Rpf1* gene, we expected that out of 932 plants about 44 (±2) plants would have the proper markers (RAPD OPO-08A$_{(1650)}$ at 1.7 cM and the SCAR marker at 3.0 cM from the gene). A total of 39 plants were identified as such and were screened for resistance against the fungus. From the 39 selected recombinant plants, 20 were resistant and 19

susceptible. These plants are currently being tested by AFLP analysis to develop a saturated map of 1.7 cM or less from the *Rpf1* gene. It is possible that since less plants were identified as recombinant than was expected from the original map, may indicate that the OPO-08A marker is slightly closer to the *Rpf1* gene than initially thought. Recombinant plants are being utilised since at a latter date we hope to eventually clone this resistance gene.

The SCAR markers confirmed the initial selection with the RAPD primers. The use of the SCAR primers to select putative resistant plants to red stele disease was greatly optimised. To test the plants with the SCAR primers in a 96 well microtiter plate, from setting up PCR to running the gel took under 4 hours. It was shown feasible to screen hundreds of plants quickly, efficiently and inexpensively. This quick prep method, when utilised in a marker-assisted selection programme, would increase the efficiency of selection of the desired plant genotype.

Marker conservation of the *Rpf1* region

The RAPD and SCAR markers linked to the *Rpf1* gene enabled that this gene could be used for pedigree analysis and in breeding programmes for marker-assisted selection of *Rpf1* genotypes (Chapter 5). Out of the 86 European and North American strawberry genotypes evaluated for the RAPD and SCAR markers only five genotypes could not be properly classified for their resistance. The *Rpf1* region was shown to be highly conserved and proved that these markers can be used in breeding programmes for the selection of the red stele (*Rpf1*) resistant genotypes.

Map-based cloning and transposon tagging

The biochemical function of many resistance genes is usually not known, molecular cloning of a gene would give greater insight into its structure and function. Map-based cloning and transposon tagging are two methods available to clone a gene on the basis of only "mutant" phenotype with an unknown product. The first plant genes cloned by map-based cloning were from *Arabidopsis* (Giraudat et al. 1992; Arondel et al. 1992). Other resistance genes have since been cloned such as against *Pseudomonas syringae* pv tomato (*Pto*) from *Lycopersicon esculentum* (Martin et al. 1993); *P. syringae* (*Rps2*) and the avirulence gene *avrRps4* from *P. syringae*pv. *pisi* to *Arabidopsis* (Staskawitz et al. 1994; Hinsch and Staskawitz 1996). Cloning should also be feasible for *Rpf1* due to the strawberry's small genome size and the availability of tightly linked flanking markers to the gene. The molecular

markers can also be used with a YAC, BAC or contig library as a starting point for chromosome walking.

Another strategy for isolating genes with unknown gene product but with an easily recognisable mutant phenotype is transposon tagging. This technique was successfully employed in maize, *Antirrhinum*, *Arabidopsis*, and petunia (Aarts et al. 1993; Bancroft et al. 1993; Chuck et al. 1993; Whitham et al. 1994; Ellis et al. 1995). The *Cf-9* resistance gene conferring resistance to a *Cladosporium fulvum* race in tomato was isolated by gene targeted transposon tagging (Jones et al. 1994). To use transposons, they are needed in many places within the genome, therefore you need a transformation and mapping system of the transposons. This technique is not feasible for the strawberry.

However, with the advancement of science alternative methods might be more feasible for cloning of the *Rpf1* gene. One such technique that might prove to be useful is a PCR based approach that has been used to isolate resistance genes in soybean (Kanazin et al. 1996; Yu et al. 1996). This was done by correlating the map positions of the PCR derived gene fragments with the resistance locus.

Strawberry transformation

One of the goals of many mapping or marker-assisted selection studies is to clone the gene and reinsert the gene into genotypes of horticultural interest. To do this, first a reliable transformation system needs to be developed. By utilising a model system (such as the diploid strawberry) many potential problems would be solved prior to working with the commercially (octoploid) genotypes. Successful transformation and regeneration of the diploid strawberry was already achieved (Chapter 6). Putatively transformed calli were selected by growth on kanamycin selection medium followed by shoot and whole plant regeneration. The presence of the genes was verified by PCR amplification, GUS histochemical analysis in conjunction with inheritance studies, and by Southern blot.

The seedlings produced from selfed transgenic plants were assessed for the number of independent loci containing inserts of the GUS gene and a two locus model of insertion was proposed. The observed (15.5:1) ratio was very close to the dihybrid ratio (15:1) expected for two independent sites of insertion into the plant genome. In comparison, James et al. (1990) determined that they either had a single insertion or a closely linked multiple insertion of the seedlings from transgenic octoploid strawberry plants.

The success of obtaining fertile diploid transgenic plants provides the foundation for future transformation experiments utilising genes of horticultural interest. Assessing the expression of these genes in the diploid strawberry would provide a basis of possible integration and expression in the octoploid cultivars.

Conclusion

The work presented in this thesis developed the framework for basic research on mapping genes in the strawberry and how to integrate the genes back into the plants genome. The development of the diploid map allowed us to work with the commercial strawberry to map the *Phytophthora fragariae* resistance gene (*Rpf1*), and the subsequent development of SCAR markers. The SCAR markers were shown to have potential use in marker-assisted selection in an applied breeding programme of commercial strawberry by their conservation in many strawberry genotypes.

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Summary

The fungus *Phytophthora fragariae*, is able to cause red stele root rot in the strawberry. Symptoms of the disease is discolouration of the stele of the roots, rotting away of the infected roots, dwarfism, wilting, and finally plant death. Chemical control of red stele with soil fumigants reduce the inoculum potential of the fungus in the soil but does not eliminate it completely. Resistant cultivars are looked upon more favourably by growers since the consumer and environmentalist are demanding the decrease of chemical treatments in cultivation.

It has previously been demonstrated that short 10 basepair length primers of arbitrary nucleotide sequence can be used to amplify segments of genomic DNA from a variety of species using PCR technology. Polymorphisms among the amplification products are typically detected as DNA segments which amplify from one parent but not from the other and can then be used to construct genetic maps. However, the dominant nature of RAPD markers does not allow the complete determination of between the two classes of homo- and heterzygous genotypes in the segregating population.

A molecular map of the diploid strawberry, *Fragaria vesca*, that consisted of five distinct linkage groups was developed. Two isozymes (PGI and SKDH) and one morphological marker (red fruit colour) were also placed on the map. The red fruit colour locus was shown to be tightly linked to both the SKDH locus and to two RAPD markers. Gene tags identified at the diploid level may help selection procedures for the commercial strawberry. Mapping of the commercial strawberry may possibly be made easier since the genetic homology between the diploid and octoploid species may extend to the linear order of genes on the chromosomes.

Classical breeding methods have already been able to incorporate resistances to various diseases in the commercial strawberry. The ability to pyramid resistance genes and accurately screen them is laborious, expensive, and at times problematic, therefore using indirect selection with molecular markers was studied. Recently, a strawberry gene-for-gene model was proposed for red stele resistance. In this study the *Rpf1* gene, out of this model, was mapped using RAPD markers. Next, specific primers (SCARs) linked in coupling phase to the gene were developed and the markers were then assessed for their conservation in European and North American genotypes.

We are the first to identify molecular markers linked with a commercially interesting trait in the strawberry. We could do so due to the disomic behaviour of the *Rpf1* region and the reliable classification into

resistance and susceptibility of the individuals of the progeny. Seven RAPD markers for *Rpf1* all had distinct presence or absence polymorphisms between the paired bulks. These markers linked to *Rpf1* were mapped to within 1.7 - 13.9 cM of the gene.

RAPD markers are difficult to reproduce and therefore, they preferentially should be converted into sequence characterised amplified region (SCAR) markers. An advantage of SCAR markers is their potential for quick and robust assessment.

A SCAR marker was constructed based upon RAPD marker OPO-16C, linked to the susceptibility allele of the *Rpf1* gene. Following the cloning and sequencing of this marker we were able to design SCAR primers specific to the alternate resistant allele. Two *Rpf1* SCAR primer sets linked to this resistance allele were constructed. These SCAR-R1 primers, when tested on the original F_1 mapping population, amplified the resistant plants only, excluding that of the same recombinants that were detected with the OPO-16 markers. The SCAR markers mapped to the same location as the original RAPD OPO-16C.

The molecular markers linked to the *Rpf1* gene were shown to be conserved in most of the European and North American genotypes. The RAPDs and SCARs are also good indicators of crossing-over events occurring in the genotypes. Out of 86 genotypes assessed, only five non-conforming genotypes were observed using the RAPD and SCAR primers. This is close to the expected number, dependent on the markers distance from the gene.

The linked markers to *Rpf1* allowed us to trace this resistance gene by pedigree analysis. Many important breeding stock materials can accurately be assessed with these markers and integrated into a marker-assisted breeding programme. These molecular markers may allow the ability to pyramid and screen for the *Rpf1* resistance gene more efficiently than present conventional methods.

The use of molecular markers for a breeding programme is limited by the number of plants from which DNA can be isolated. Therefore, a DNA mini-preparation method was developed with which a single individual can isolate over 200 samples/day. This DNA isolation technique eliminates time-consuming and non-critical steps such as extensive grinding, ethanol washes, and RNAse treatment.

The ability to integrate useful genes directly into genotypes of horticultural interest is one of the final goals of many studies, therefore a reliable transformation and regeneration system needs to be available. The diploid strawberry was used as model system for this research prior to working with the octoploid strawberry due to the less complex nature of its

genome. The diploid strawberry was successfully transformed using *Agrobacterium*-mediated transformation. Primary (R0 generation) transformants were vegetatively propagated to produce five R0 runner plants which were allowed to self-pollinate to produce the R1 generation seedlings. When R1 generation seedlings were tested by GUS histochemical analysis, the segregation data fit a 15:1 ratio ($0.5>p>0.25$), indicating the independent segregation of two transgene insertion loci.

To summarise, the diploid strawberry was first utilised as a model system to construct a molecular map prior to working with the commercial strawberry. In the octoploid strawberry a red stele disease resistance gene, *Rpf1*, was mapped with seven RAPD markers and highly specific SCAR markers were constructed to this gene. The RAPD and SCAR markers were assessed in 86 genotypes and were shown to be conserved in regard to the *Rpf1* region. The diploid strawberry was used as a model system to develop a reliable transformation method to integrate genes back into the plant and stabily passed into the progeny plants. The research presented in this study has opened the door for further work of mapping other genes of interest in the strawberry to help breeders in the future and to reduce pesticide inputs in this crop.

Samenvatting

De schimmel *Phytophthora fragariae* is de veroorzaker van roodwortelrot in aardbei. De symptomen van de ziekte zijn ontkleuring van het wortelmerg, wegrotten van de geïnfecteerde wortels, dwerggroei en verwelking wat uiteindelijk leidt tot het afsterven van de plant. Chemische behandeling van roodwortelrot met een fungicide zorgt dat het inoculum potentieel van de schimmel vermindert doordat de schimmel de plant niet kan ingroeien. De schimmel blijft wel aanwezig in de bodem. Bij aardbeitelers en milieubeschermers wordt de vraag naar resistente cultivars steeds groter, omdat een verminderd gebruik van chemische bestrijdingsmiddelen verplicht is.

Het is bekend dat korte, 10 basepaar lange, primers met een willekeurige nucleotidevolgorde kunnen worden gebruikt om met PCR technieken DNA segmenten in een groot aantal soorten te amplificeren. Polymorfismen tussen planten worden gedetecteerd doordat er DNA segmenten geamplificeerd worden bij slechts één ouder. Dergelijke verschillen kunnen worden gebruikt voor het maken van genetische kaarten. Door de dominante aard van de RAPD-merkers is het echter onmogelijk om een indeling te maken met meer dan twee klassen , er zijn echter drie klassen genotypen mogelijk bij de planten in een nakomelingenpopulatie.

Van de diploïde aardbei *Fragaria vesca* is een moleculaire kaart ontwikkeld met daarop vijf duidelijk te onderscheiden kopplelingsgroepen. Twee isozymen PGI en SKDH en een morfologische merker (rode fruitkleur) zijn ook op de kaart gezet. De plaats van de fruitkleurmerker laat zien dat er een sterke koppeling bestaat tussen deze merker en het SKDH-locus en twee RAPD-merkers. Moleculaire merkers die op het diploïde niveau worden geïdentificeerd kunnen bijdragen aan verbetering van de selectieprocedures voor de commerciële aardbei. Het maken van een genetische kaart van de commerciële aardbei kan mogelijk eenvoudiger indien de kennis van genetische homologie tussen de diploïde en de octoploïde soorten kan worden uitgebreid.

Met klassieke veredelingsmethoden zijn al een aantal ziekteresistenties in commerciële aardbeirassen geïncorporeerd. De methoden om verschillende resistentiegenen te combineren zijn tijdrovend, kostbaar en soms problematisch. Daarom zijn de mogelijkheden onderzocht om indirecte selectie met moleculaire merkers hiervoor te gebruiken. Kort geleden is een gen-om-gen model gepresenteerd voor roodwortelrotresistentie. In het hier beschreven onderzoek is één van de betrokken genen, het *Rpf1* gen, in kaart gebracht door gebruik te maken van RAPD-merkers. Van hieruit zijn specifieke primers (SCAR's) voor deze

merker ontwikkeld en beproefd op de stabiliteit van de koppelingmerker en gen in Europese en Noordamerikaanse genotypen.

Wij waren in staat moleculaire merkers te identificeren voor commercieel interessante eigenschappen van de aardbei. We maakten hierbij gebruik van het disome gedrag in de *Rpf1*-regio en de betrouwbare classificatie met betrekking tot resistentie en vatbaarheid van de individuele planten. Zeven RAPD merkers werden gevonden door gebruik te maken van het wel of niet aanwezig zijn in groepen van resistente respectievelijk vatbare planten. Deze aan *Rpf1* gekoppelde merkers werden in kaart gebracht en hadden een afstand van 1.7 - 13.9 cM tot het gen. RAPD-merkers zijn moeilijk te reproduceren en ze zouden bij voorkeur omgezet moeten worden naar Sequence Characterised Amplified Region (SCAR) merkers. Het voordeel van SCAR merkers is dat ze bruikbaar zijn voor snelle en betrouwbare bepalingen.

Van de RAPD-merker OPO-16C, die gekoppeld is met het vatbare allel van het *Rpf1* gen, werd een SCAR merker gemaakt. Door het kloneren en het sequencen van deze merker waren we in staat om SCAR primers te ontwerpen die het resistente allel konden aantonen. Twee *Rpf1* SCAR "primer sets" die het resistente allel kunnen aantonen werden geconstrueerd. Bij het testen op de oorspronkelijke F_1 populatie werd alleen DNA van resistente planten geamplificeerd en planten met een bekende overkruising tussen merker en gen.

De koppeling tussen moleculaire merkers en het *Rpf1* gen is behouden gebleven in de meeste Europese en Noordamerikaanse genotypen. Het voorkomen van overkruising in een aantal genotypen kon goed met de RAPD's en SCAR's worden aangetoond. Onder de 86 bepaalde genotypen werden met behulp van RAPD- en SCAR primers slechts vijf afwijkende genotypen waargenomen. Dit komt overeen met het theoretisch verwachte aantal op basis van de afstand tussen de merker en het gen.

De aan het *Rpf1* gen gekoppelde merkers gaven ons de mogelijkheid dit resistentiegen te traceren met behulp van stamboomonderzoek. Voor veel belangrijke lijnen kan nauwkeurig de aanwezigheid van het resistentiegen met deze merkers bepaald worden en deze kennis kan in veredelingsprogramma's worden gebruikt. Met deze moleculaire merkers kan voor het resistentie *Rpf1* gen efficiënter worden gescreend dan met de conventionele methoden.

Het gebruik van moleculaire merkers in een veredelingsprogramma wordt beperkt door het aantal planten waaruit DNA kan worden geïsoleerd. Er werd daarom een mini-preparatiemethode ontwikkeld waarmee één persoon meer dan 200 monsters per dag kan isoleren. Met deze DNA-isolatietechniek hoeven geen tijdrovende en andere handelingen zoals

fijnmalen, wassen met ethanol, RNAse behandeling, meer uitgevoerd te worden.

De mogelijkheden om bruikbare genen direct te integreren in tuinbouwkundig interessante genotypen is een einddoel van veel studies. Er moet hierom een betrouwbaar transformatie- en regeneratiesysteem beschikbaar zijn. Door de minder complexe aard van haar genoom is eerst de diploïde aardbei onderzocht, voordat aan de meer complexe octoploïde aardbei werd begonnen. De diploïde aardbei werd met succes getransformeerd met behulp van een *Agrobacterium* transformatie. De primaire (R0 generatie) transformanten werden vegetatief voortgeplant met vijf R0 uitlopers die vervolgens na zelfbevruchting de R1 generatie zaailingen produceerden. Met de GUS histochemische analyse methode kon de onafhankelijke splitsing van de twee transgene insertie loci worden aangetoond, waarbij de afzonderlijke data passen bij een 1:15 verhouding ($0.5>p>0.25$). Deze onderzoeksmethode op het diploïde niveau kan waarschijnlijk worden geëxtrapoleerd naar de octoploïde planten, omdat beide varianten grote overeenkomsten vertonen.

Samenvattend: de diploïde aardbei werd eerst gebruikt als modelsysteem voor de constructie van een genetische kaart. Daarna werd gewerkt aan een methode om het resistente *Rpf1* gen tegen roodwortelrot bij commerciële aardbeien in kaart te brengen. Er werd hierbij gebruik gemaakt van zeven RAPD-merkers en zeer specifieke SCAR merkers die voor het aantonen van dit gen werden geconstrueerd. Met deze RAPD- en SCAR-merkers werden 86 genotypen gescreend en er werd aangetoond dat de merkers geconserveerd waren in het *Rpf1* gebied. De diploïde aardbei werd als modelsysteem gebruikt om een betrouwbare transformatiemethode te ontwikkelen waarmee genen stabiel kunnen worden geïntegreerd in het nageslacht. Het hier gepresenteerde onderzoek is een aanzet tot het verder in kaart brengen van andere interessante genen in de aardbei wat uiteindelijk kan leiden tot een beter produkt en een verminderd gebruik van chemische bestrijdingsmiddelen.

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Related Publications

Haymes KM (1993) Genome Mapping and *Agrobacterium*-mediated transformation of the diploid strawberry. M.S. Thesis, University of New Hampshire, Durham, NH 03824.

Davis TM, **Haymes KM** (1993) Genetic mapping of the diploid strawberry using Random Amplified Polymorphic DNA (RAPD) Markers. Acta Horticulturae 348:439.

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Curriculum Vitae

Kenneth Michael Haymes was born on the 31 March 1966 in Hauppauge, New York, USA. He graduated with a degree in biology from the State University of New York at Oneonta in 1988. The last semester of his univerisity study was spent at an international research center called the William H. Miner Agricultural Research Institute where he was in the program of in vitro cell biology and biotechnology: The area of his specialization was in genetics and biotechnology. After his university studies he worked in a hospital laboratory, Department of Nuclear Medicine, in Baltimore, Maryland. From September 1990 till December 1992 he studied for his MSc degree in Plant Biology at the University of New Hampshire. In January 1993, he successfully defended his thesis entitled "Genome Mapping and *Agrobacterium*-mediated transformation of the diploid strawberry". From October 1993 till December 1996 he worked as a researcher in the Department of Vegetable and Fruit Crops of the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO) in Wageningen (The Netherlands). The area of research was on resistance gene mapping, cloning and the development of specific markers to the the fungal disease *Phytophthora fragariae* var. *fragariae* in the commercial strawberry.

The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveller, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

by: Robert Frost