Abstract

McCord, Per Hilding. Sinks above and sinks below— physical mapping of fruit-derived ESTs in peach, a model species for *Prunus* and Rosaceae structural genomics, and identification of amplified fragment length polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids (Under the direction of Bryon R. Sosinski and G. Craig Yencho).

Peach (*Prunus persica* (L.) Batsch) has been proposed as a model organism for structural genomics in the family Rosaceae. Several resources have been or are currently being developed to study the structural genomics of the species. A highly saturated genetic linkage map has been developed from an interspecific 'Texas' almond X 'Earligold' peach cross (T X E), and two peach BAC libraries from the cultivars 'Nemared' and 'Lovell' have been generated for the creation of a physical mapping resource. A framework map has been generated by the hybridization of markers from the T X E and other *Prunus* maps to the 'Nemared' library. In addition, peach and almond unigenes are being hybridized to the library in order to generate an anchored transcript map, identify contigs for overall map construction, and provide information on genome organization. A subgroup of the *Prunus* unigene set comprising 942 ESTs derived from peach fruit mesocarp was hybridized to the 44,150-clone 'Nemared' library. Eight hundred ninety-nine of these ESTs successfully hybridized to the library, identifying 3475 BACs, of which 2725 were unique. Seventy-six of these ESTs are now anchored to the reference map by hybridization to genetically anchored BAC clones or contigs. Twenty-six of these ESTs mapped near loci for important agronomic traits, including flesh acidity, fruit skin pubescence, and almond shell hardness. Unanchored ESTs generated contigs of 2 to 44 clones, which are being

fingerprinted and integrated into the growing peach physical map in preparation for sequencing of the peach genome.

Internal heat necrosis (IHN) is a physiological disorder of potato tubers resulting in the discoloration of parenchymal tissue. Environmental conditions and the large acreage of the IHN-susceptible cultivar 'Atlantic' result in significant losses due to the disorder in the mid-Atlantic states and Florida. A combination of bulked segregant analysis and AFLP marker technology was used to search for molecular markers for IHN in a population of tetraploid 4x-2x *S. tuberosum* x *S. phureja-S. stenotomum* (*tub* x *phu-stn*) hybrids. These clones are being used to breed an IHN-resistant, high specific-gravity replacement for 'Atlantic'. One marker, potPCR13-HindIII-R, was identified in two small test populations and was strongly associated with resistance to IHN, explaining 69.9% and 64 % of the observed variation for IHN incidence and severity, respectively. This marker showed strong sequence homology to calcium-dependent protein kinases (CDPKs), reinforcing prior evidence of the importance of calcium to the manifestation of IHN. When tested on a different set of clones from a combining ability study for IHN, this marker was no longer significant. However, a second putative marker from the BSA-AFLP screen (potPCR31-A) that was not statistically significant in the small populations, was significantly associated with IHN susceptibility in this second population, and the regression models of potPCR-31-A on IHN severity and incidence explained 17.8% and 18.6% of the phenotypic variation, respectively. This marker showed homology to plastid terminal oxidases (PTOXs), which are involved in desaturation of carotenoids, and may have a role in protecting biomolecules under oxidative stress. The small sample sizes used in marker development and initial testing may have resulted in these markers being significant predictors only in certain populations. It is also possible that, given the quantitative nature of IHN, only a subset of all

IHN-associated loci may be necessary for the disorder to be manifest. This research is the first molecular marker study of the genetics of IHN, and has set a foundation for future studies.

Sinks above and sinks below— physical mapping of fruit-derived ESTs in peach, a model species for *Prunus* and Rosaceae structural genomics, and identification of amplified fragment length polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids

by

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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

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Biography

Per Hilding McCord was born on 1 August 1976 in Portland, Oregon. He is the oldest of nine children, eight boys and one girl. His parents moved to his mother's family homestead in the forests outside Seaside, Oregon in October 1979, where they currently reside. Per's interest in plants was piqued by growing up in the middle of nature, and by frequent visits with his nearby maternal grandmother, who is an avid gardener. Reading about Luther Burbank, and later the principles of genetics and DNA, cemented his desire to become a plant geneticist/breeder. Per attended elementary school in nearby Cannon Beach, and middle and high school in Seaside. Following his graduation from high school in 1994, he attended Brigham Young University (BYU) in Provo, Utah for one year. He then served a two-year proselyting mission in Pennsylvania and New Jersey for the Church of Jesus Christ of Latter-day Saints, where he truly "grew up".

Per continued his studies at BYU after his mission, graduating with a B.S. in Plant Genetics and Breeding in December 2000. From February to July 2001 he worked as a research assistant at Cornell's New York State Agricultural Experiment Station in Geneva, New York, where he successfully cloned partial sequences of several anthocyanin pathway genes from raspberry. Per married Julie Ann McNulty on 4 August 2001 in Utah. They returned to New York where Per worked at Cornell on the Ithaca campus, in the Department of Plant Breeding's pepper breeding and genetics program. In March 2002 he was accepted into the graduate program at North Carolina State University, Department of Horticultural Science. His research toward a Master of Science degree has been under the advisement of Bryon R. Sosinski and G. Craig Yencho from the Department of Horticultural Science, and Rebecca S. Boston from the Department of Botany.

Table of Contents

List of Tables v

List of Figures vi

List of Abbreviations and Symbols viii

Preface xii

Chapter One: The development and future of peach [*Prunus persica* (L.) Batsch] as a model organism for the family Rosaceae 1

- Abstract 3
- The importance of model organisms in genome research 5
- Peach and the family Rosaceae 8
- Molecular markers and linkage mapping 9
- Interspecific T X E map—General reference map 10
- EST Database and the Genome Database for Rosaceae (GDR) 12
- Physical/Transcript Mapping 15
- The *evergrowing* locus 17
- Summary and Conclusion 18
- References 22
- Tables and Figures 31

Chapter Two: Physical mapping of fruit-derived ESTs from peach, a model species for *Prunus* and Rosaceae structural genomics 37

- Abstract 38
- Introduction 39
- Materials and Methods 40
  - Membrane processing and EST probe labeling 40
  - Probe hybridization and data collection 42
- Results and Discussion 44
- Summary and Conclusion 47
- References 49
- Tables and Figures 51

Chapter Three: Internal heat necrosis of potato—a review 58

- Abstract 59
- Introduction 60
- Temperature and moisture 61
- Soil Fertility/The Calcium Connection 64
- Genetics 67
- Summary and Conclusion 69
- References 71

Table of Contents-- continued

Chapter Four: Identification of Amplified Fragment Length Polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids 74

Abstract 77
Introduction 79
Materials and Methods 82
Field evaluation of IHN populations 82
AFLP generation and conversion 83
Marker development 84
Marker testing 85
Results 86
Discussion 87
References 92
Tables and Figures 96

Appendix
Peach ESTs anchored to physical map 101

List of Tables

Review: The development and future of peach [*Prunus persica* (L.) Batsch] as a model organism for the family Rosaceae

Table 1. Linkage maps published for peach and other *Prunus* species. 31

Table 2. Description of major genes (21) and QTL (28) affecting morphological or agronomic characters in peach that can be placed on the *Prunus* reference map. 35

Physical mapping of fruit-derived ESTs from peach, a model for *Prunus* and Rosaceae structural genomics

Table 1. Summary of peach EST hybridization results. 51

Identification of Amplified Fragment Length Polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids.

Table 1. Putative markers identified for IHN resistance/susceptibility using the BSA-AFLP technique. 99

Table 2. Final regression models for the markers tested on members of the combined IHN populations 1 and 2 (A), and for the IHN combining ability study (B). 99

List of Figures

Review: The development and future of peach [*Prunus persica* (L.) Batsch] as a model organism for the family Rosaceae

Figure 1. The current (January 2005) *Prunus* reference map. 34

Physical mapping of fruit-derived ESTs from peach, a model for *Prunus* and Rosaceae structural genomics

Figure 1. Diagrams outlining spotting pattern of BAC clones (upper right), and the decoding of their location on 6-field arrays (left). 52

Figure 2. Diagrams outlining spotting pattern (lower right, identical to Figure 1) and the decoding of their location on 4-field arrays. 53

Figure 3. Example of BAC membrane hybridized with 10 EST probes. 54

Figure 4. Typical results following hybridization of positive clones from BAC library to individual ESTs. 55

Figure 5. Histogram of the numbers of BAC clones that hybridized to EST probes. 56

Figure 6. The ten BAC clones that hybridized to the most EST probes. 57

Identification of Amplified Fragment Length Polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids.

Figure 1. Internal heat necrosis in potato. 96

Figure 2. Ratings of IHN incidence of members of IHN Population 1, the population used for BSA. 97

Figure 3. Ratings of IHN incidence of members of IHN 2, the population used for preliminary marker testing. 97

Figure 4. Histogram of the range of IHN incidence in the IHN combining ability progenies. 98

Figure 5. Histogram of IHN incidence amongst members of family B2460, which had the highest incidence of IHN. 98

List of Figures- continued

Figure 6. The marker potPCR13-HindIII-R, tested on members of IHN Populations 1 and 2. 100

Figure 7. Marker potPCR31. 100

Appendix

Peach ESTs successfully anchored to the physical map. 101

List of Abbreviations and Symbols

AFLP	amplified fragment length polymorphism
B.S.	Bachelor of Science
BAC	bacterial artificial chromosome
BC1	backcross, first generation
BLAST	basic local alignment search tool
BSA	bulk segregant analysis
BYU	Brigham Young University
CART	classification and regression tree
CDPK	calcium-dependent protein kinase
cM	centiMorgan
CS	conventional selection
CTAB	cetyltrimethylammonium bromide
Cu	copper
CUGI	Clemson University Genome Institute
cv.	cultivar
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxy (A,C,G, or T)-triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid

List of Abbreviations and Symbols- continued

EST	expressed sequence tag
evg	*evergrowing*
Fe	iron
FPC	FingerPrinted Contigs
G X E	genotype by environment
GDR	Genome Database for Rosaceae
H	heritability (broad-sense)
IBS	internal brown spot
IHN	internal heat necrosis
IMA	intermicrosatellite amplification
K	potassium
kb	kilobase(s)
LB	Luria-Bertani
lsd	least significant difference
M	molar
MADS	MCM1, AGAMOUS, DEFICIENS, SRF
MAS	marker assisted selection
Mb	megabases
Mg	magnesium
mJ	millijoules
mM	millimolar
Mn	manganese

List of Abbreviations and Symbols- continued

N	normal
Na	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogram
P	phosphorus
PCR	polymerase chain reaction
PTOX	plastid terminal oxidase
QTL	quantitative trait locus/loci
RAG	resistance associated gene
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RGA	resistance gene analog
RNA	ribonucleic acid
RNAi	RNA interference
RosaGEC	Rosaceae Genomics, Genetics and Breeding Executive Committee
S	sulfur
SAS	Statistical Analysis Systems
SCAR	sequence characterized amplified region
SDS	sodium dodecyl sulfate
SG	specific gravity

List of Abbreviations and Symbols- continued

SNP	single nucleotide polymorphism
SSC	saline sodium citrate
SSR	simple sequence repeat
STS	sequence tagged site
subsp.	subspecies
T X E	Texas' by 'Earligold'
Taq	*Thermophilus aquaticus*
Tris-HCl	tris-(hydroxymethyl)aminomethane hydrochloride
USDA	United States Department of Agriculture
UTR	untranslated region
UV	ultraviolet
WGS	whole genome shotgun
YAC	yeast artificial chromosome
Zn	zinc
µCi	microcurie(s)
µg	microgram(s)
µL	microliter(s)
µM	micromolar

Preface

The research presented is of two separate projects, comprising basic research of the structural genomics of peach, and applied research towards breeding in potato. Two quite different projects were chosen because the methods and techniques involved in each project complemented one another, resulting in a more educational, rewarding research and educational experience.

The physical mapping of peach ESTs was part of a large-scale collaborative effort to place 3000 peach and almond ESTs on the developing peach map. Peach is a model species for the large Rosaceae family, and in the course of research I was able to interact with researchers around the world involved in a wide variety of interesting research endeavors. The research took place entirely in the laboratory. Here I learned the techniques involved in high-density BAC filter preparation and processing using modern high-throughput equipment, in addition to radiolabeling and hybridization of DNA. Managing and interpreting the data generated by the experiments greatly enhanced my ability to work with image software and large datasets. These techniques and tools will serve me well in a career focused in the laboratory.

Much of the work to develop molecular markers for internal heat necrosis (IHN) in potato was also laboratory-based. I received valuable exposure to basic principles of bioinformatics, and increased my proficiency in such techniques as PCR primer design, cloning, and sequencing. However, this project also had a significant field component. In the field I interacted with growers and gained first-hand knowledge of the basics of selection criteria for breeding and basic potato production. Since I also hope to become a plant breeder, the field component of the potato research has been invaluable in providing first-hand experience in the art as well as science of breeding.

This combination of laboratory and field work has strengthened and enriched my educational experience as a master's student. Interaction with specialists in different species has also been beneficial by exposing me to a wider range of research questions and potential solutions, expanding my 'sphere of reference' when considering a given problem. Though it has been extra work doing two distinct research projects, the rewards have been worth the effort.

Chapter One

The development and future of peach [*Prunus persica* (L.) Batsch] as a model organism for the family Rosaceae

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(In the format appropriate for submission to Tree Genes and Genomes)

The development and future of peach [*Prunus persica* (L.) Batsch] as a model organism for the family Rosaceae

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Abstract

Model organisms play a crucial role in genetic and genomic studies. Peach [*Prunus persica* (L.) Batsch] is an ideal model organism for structural genomics in the important family Rosaceae, primarily due to its small genome and short juvenility phase. International collaboration has produced and is continuing to develop a number of valuable resources for genomics studies in peach. A saturated linkage map from an interspecific almond X peach cross has been developed, resulting in a common linkage group terminology and a set of core markers that can be used to anchor other maps within the genus *Prunus*. Anchoring these maps has permitted a number of important traits and QTL to be positioned on this reference map. This map has also been used for comparative mapping within *Prunus* (near-total collinearity), with apple (significant collinearity with some rearrangements), and with *Arabidopsis* (conservation of very small linkage blocks, but extensive large-scale rearrangements). Clones from a 'Nemared' peach BAC library have been genetically anchored by hybridization with markers from the reference map to generate a framework physical map, which is being constructed through a combination of marker/probe hybridization and clone fingerprinting. A growing set of unigenes, currently numbering over 3800 members, has been identified from peach and almond ESTs, and these are being hybridized to the BAC library to genetically anchor candidate genes, generate additional contigs, and provide important information on gene organization. The chromosomal region containing the '*Evergrowing*' locus has been cloned and sequenced, using the 'Nemared' BAC library as an important tool. This achievement, combined with the recent development of a reliable method for stable transformation of peach, will now allow this important regulator of dormancy induction to be identified and characterized. With the physical map

nearing completion, and the availability of a hybrid clone-by-clone/whole genome shotgun sequencing strategy amenable to large-scale collaboration, peach is in an excellent position to be one of the first tree genomes sequenced.

The importance of model organisms in genome research

Since the time of Gregor Mendel, model organisms have been important for the study of genetics. Mendel chose the garden pea, *Pisum sativum*, for his experiments due to its ease of cross-pollination, the existence of a number of discontinuous or qualitative traits, and its short life cycle (Tamarin 1996). The last two traits, in particular, have been features of most of the model organisms employed since that time. In Mendel's case, his use of a model organism was a key element of his work, which led to the formulation of the modern foundational theories of heredity, the rules of segregation and independent assortment.

Model organisms are useful to the study of genes and genomes for two reasons. The first is rather pragmatic-- relative ease of use. As mentioned previously, most model organisms have a range of easily observable phenotypes and rather short life cycles. Other important features relative to the utility of a model organism include small size, high numbers of progeny, a disomic pattern of inheritance, and a small genome. Each of these elements allows genetic and genomic experiments to be performed more easily and on a larger scale compared to less amenable organisms. In particular, a disomic pattern of inheritance and a small genome facilitate the analysis of data from these experiments.

For example, the fruit fly *Drosophila melanogaster* was one of the first model organisms of the 20th century. *Drosophila* is small, completes its life cycle in approximately two weeks (Tamarin 1996), and produces large numbers of offspring (females each lay several hundred eggs). This means that many large populations can be reared in a small space, and in a short amount of time. In addition, it only has five chromosomes. These advantages undoubtedly contributed to the early use of *Drosophila* in genetics experiments. The classic genetic linkage map of *Drosophila* was the first published for any organism.

Research on *Drosophila* led to the development of the chromosomal theory of inheritance, and the important tools of cytogenetic markers, genomic libraries, positional cloning, and integrative transgenesis (reviewed in Rubin and Lewis 2000).

In the plant kingdom, the potential usefulness of the cress *Arabidopsis thaliana* was initially noted by Laibach (reviewed in Somerville and Koorneef 2002). Like *Drosophila*, *Arabidopsis* has the advantages of small size, large progenies, and short life cycle, and is a diploid with a small chromosome number (x = 5) and small genome (157 Mb, Bennett et al. 2003). However, crop plants such as maize were the focus of most plant genetics research until the 1980s, when the power of new molecular techniques, especially when used with small genomes, became more apparent (reviewed in Somerville and Koorneef 2002). DNA-based molecular markers such as RFLPs and SSRs have facilitated the creation of a highly saturated (50,000 polymorphisms) genetic map for *Arabidopsis*, which enables the positional cloning of new mutants. Large-insert genomic libraries such as YACs, BACs, and phagemids allowed for efficient generation of deep-coverage genomic libraries with a relatively small number of clones. The combination of these and other techniques (PCR, transposon tagging, gene and enhancer traps, etc.) and an organism with the above-mentioned characteristics led to the preeminence of *Arabidopsis* amongst genetic and genomic studies of higher plants. Studies in *Arabidopsis* have led to a much greater understanding of many facets of plant development and physiology, including (but certainly not limited to) floral patterning (reviewed in Weigel and Meyerowitz 1994), meristem maintenance and organogenesis (Schoof et al. 2000, Baürle and Laux 2003), and the organization, polarity, and shape of roots and leaves (Byrne et al. 2000, Nakajima et al. 2002, Emery et al. 2003, Eshed et al. 2004). *Arabidopsis* was the first plant to have its genome

sequenced (*Arabidopsis* Genome Initiative 2000), and a goal has been set to determine the function of every gene in *Arabidopsis* by 2010 (The 2010 Project, NSF 2001). This latest project has been made more feasible by the ease with which this species can be transformed (Clough and Bent 1998), another useful feature of model organisms.

The second raison d'être of a model organism is its intrinsic importance to society, and/or the applicability of information gained from its investigation to other important species. As important as *Arabidopsis* has been to the study of plant genomes, it has virtually no economic value unique genes or traits discovered in this species do not provide rapid economic benefits. Although some degree of synteny has been preserved between *Arabidopsis* and more economically important species (Dominguez et al. 2003), there is ample evidence of extensive reorganization of genomes, making comparative structural genomics more difficult (Gebhardt et al. 2003). Additionally, in the course of evolution, gene duplications have been followed by subtle but important differentiation (Kolukisaoglu et al. 2004), so that putative homologs and/or orthologs between *Arabidopsis* and other species may actually have quite different characteristics (Sorefan et al. 2003). Finally, there are multiple examples of species-specific genes, important to the value of these species, that are not found in *Arabidopsis.* Examples include an alcohol acyltransferase from strawberry implicated in aroma development during ripening (Aharoni et al. 2000), a number of un-characterized proteins involved in Gerbera flower development, a member of the large Asteraceae family (Luitenen et al. 2005), and more than 20 genes preferentially expressed during early and latewood formation in loblolly pine (Egertsdotter et al. 2004)All of these factors limit to some extent the transfer of information obtained from *Arabidopsis* to other important species.

Peach and the Family Rosaceae

In temperate regions, one of the most economically important plant families is the Rosaceae (Rajapakse et al. 1995). The family Rosaceae includes species that supply food (strawberry, bramble, apple, pear, stone fruits, and almond), timber (black cherry), and ornamentation (rose, quince, hawthorn, weigela, and many others). Worldwide, 2002 production of peaches and nectarines alone was estimated at 13.5 million metric tons (FAOSTAT 2002). The value of U.S. production of Rosaceae fruits and nuts for 2003 was valued at over $6.3 billion, over half the total value of non-citrus fruits and nuts, and the U.S. wholesale value of roses as cut flowers for 2002 was valued at nearly $247 million (USDA-NASS 2004, USDA-ERS 2003).

Despite its economic importance, the family Rosaceae has, until the last decade, been the focus of relatively little genetic and genomic research. Although the family as a whole has great economic value throughout the world, individual species are only locally important. In addition, large genomes, polyploidy and a long juvenility period for many Rosaceae members have made them difficult to study genetically (Georgi et al. 2002). Instead of a single model organism for the family, which is the case in the Brassicaceae (*Arabidopsis*) and Fabaceae (*Medicago trunculata*—www.medicago.org), the U.S. Rosaceae Genomics, Genetics and Breeding Executive Committee (RosaGEC) has proposed a three-species model 'system' for Rosaceae. The committee has proposed that apple serve as the Rosaceae model for functional genomics, due to the wealth of expressed sequence data already available in this species. Due to its small size, short life cycle and ease of transformation, the diploid strawberry *Fragaria vesca* has been proposed as a model for translational genomics. Peach, the focus of this review, is to be the model for structural genomics studies. The benefits of

using three different species within the Rosaceae for development of a model system stem from the diversity of the family, as each of these species has its own advantages (RosaGEC 2005).

The peach, *Prunus persica* L. Batsch, is an ideal model organism for structural genomic studies for the family Rosaceae (Abbott et al. 2002). Like many other model organisms, it has a relatively short 'life-span', in this case a juvenility phase from germination to flowering of 2-3 years, which is low for a tree species (Abbott et al. 2002, Horn et al. 2005). It is a diploid (2n = 2x = 16), with a genome size of approximately 300 Mb, about twice that of *Arabidopsis*. Peach is the best genetically characterized species of the family (Mowrey et al. 1990), with numerous single-gene traits and quantitative trait loci (QTL) documented (Bailey and French 1949, Scorza and Sherman 1996, Dirlewanger and Arús 2004). Furthermore, it can be clonally propagated, allowing populations to be maintained indefinitely (Dirlewanger et al. 2004a), and peach combines many important characteristics into one organism, including (but not limited to) wood formation, bud dormancy, and fleshy, edible fruits that undergo distinct changes during ripening.

Molecular markers and linkage mapping

Generally, the first major step in genetic and/or genomic characterization of an organism is the development of a linkage map. Table 1 lists the linkage maps published for peach and several other closely related *Prunus* species, with a summary of their characteristics. Due at least in part to the narrow genetic base of cultivated peach (Scorza et al. 1985), and resultant low genetic diversity, linkage maps developed before the advent of DNA-based molecular markers were extremely small. As can be seen from the table, DNA-based markers greatly improved the length and marker saturation of later peach linkage

maps. However, as previously mentioned, peach has a low level of polymorphism, which limits the amount of marker saturation obtainable in any given map, even when using molecular markers. All of the intraspecific maps in Table 1 contained more linkage groups than chromosomes and/or groups with large regions devoid of markers. A saturated linkage map is important for the identification and cloning of important genes, including QTL. Markers from a saturated map may also be used to generate maps from other crosses. The use of previously mapped markers in new populations allows for studies of macro- and microsynteny between species, known as comparative mapping (Dirlewanger et al. 2004a).

Interspecific T X E map—General reference map

One of the hallmarks of research surrounding model organisms is collaborative effort. In 1993, a European consortium of laboratories began efforts to develop a general reference linkage map for *Prunus* (Arús et al. 1994). Such a map would prove valuable not only for peach, but for other important members of the genus, including almond (*P. dulcis*), apricot (*P. armeniaca*), sweet cherry (*P. avium*), sour cherry (*P. cerasis*), and plum (*P. domestica and P. salicina*). In addition, this map would be useful for comparative mapping for other members of the Rosaceae, and even other higher taxa.

The results of these collaborative efforts from six European laboratories in four countries (France, Italy, and Spain) were published by Joobeur et al. (1998); these results are summarized in bold in Table 1. An interspecific cross (almond cv. 'Texas' X peach cv. 'Earligold') was used as a mapping population because of its high intrinsic polymorphism[1], and because it provides for simultaneous comparative and linkage mapping. To allow for

[1] Although crosses between highly interfertile species have the potential advantage of more polymorphisms, they can also exhibit segregation distortion and compression of map distances. Segregation distortion was reported by the authors.

maximum portability of markers from this map to others, only easily transferable RFLP and isozyme markers were used. To strengthen the position of this map as a core reference for *Prunus* and even the Rosaceae, RFLP probes from almond, *P. ferganensis*, cherry, plum, and apple were used in addition to peach. The T X E map upon its initial publication (Joobeur et al. 1998) contained 213 markers (isozymes and RFLPs) spanning 491 cM.

SSR markers for *Prunus* were not widely available to Joobeur et al., but have since been identified in peach by Cipriani et al. (1999), Sosinski et al. (2000), Testolin et al. (2000) Dirlewanger et al. (2002a), and Aranzana et al. (2002). Aranzana et al. (2003) used these SSRs from peach and other *Prunus* species to add 96 markers to the original map. Dominguez et al. (2003) added 123 RFLP-based markers from *Arabidopsis*, and Dirlewanger et al. (2004a) contributed 89 more SSRs and five STS (sequence tagged site) markers. Currently, the T X E map (Figure 1) contains 562 markers covering 519 cM, a density of 0.92 cM per marker (Dirlewanger et al. 2004a). The T X E map is now considered the reference genetic linkage map for *Prunus* (Horn et al. 2005). The high level of saturation of the T X E map has made it useful for the new technique of selective or bin mapping (Vision et al. 2000). This method involves selecting a subset of individuals from the original mapping population. Individuals are selected which contain a sufficient number and distribution of recombination events and therefore divide the linkage groups or chromosomes into a number of small intervals, or bins. These individuals can then be used to test and map additional markers with a high degree of accuracy (defined by bin size), without having to genotype the entire mapping population. Several hundred new SSR markers have been placed on the T X E map with the bin mapping technique, further increasing the saturation of the map (P. Arús, personal communication).

Markers from this map have been successfully used to anchor many additional maps from various *Prunus* species, including almond (Joobeur et al. 2000, Jáuregui et al. 2001, Bliss et al. 2002), apricot (Lambert et al. 2003), Myrobalan plum (*P. cerasifera*, Dirlewanger et al. 2004b), *P. davidiana* (Viruel et al. 1998, Foulongne et al. 2003a), *P. ferganensis* (Dettori et al. 2001), peach (Viruel et al. 1998, Dettori et al. 2001, Jáuregui et al. 2001,Yamamoto et al. 2001, Bliss et al. 2002, Etienne et al. 2002, Foulongne et al. 2003a, 2003b) and sweet cherry (Dirlewanger et al. 2002b). The populations for these new maps often segregate for traits not segregating in the reference map. Linkages detected between these traits and 'reference' markers (or new markers linked to them) have allowed loci for a number of important agricultural traits to be identified on the original reference map (Dirlewanger et al. 2004a). Examples of such traits include tree architecture, fruit quality, and disease resistance (Table 2).

Transferable markers and a consensus linkage group terminology have also facilitated comparative mapping in the Rosaceae between the T X E reference map and the previously mentioned maps, as well as an earlier peach map (Rajapakse et al. 1995) and a linkage map from apple, *Malus domestica* (L.) Borkh (Dirlewanger et al. 2004a). These comparisons have demonstrated an extremely high degree of synteny amongst these members of the Rosaceae. One interesting result from these studies, and a departure from the overall synteny observed, is a reciprocal translocation between linkage groups 6 and 8 (Jáuregui et al. 2001, Yamamoto et al. 2001). This breakpoint likely originated in the peach germplasm, and is in the vicinity of the leaf color locus (green/red) *Gr*, which may serve as a marker for the translocation. Comparison between the T X E genetic map and *Arabidopsis* physical map (Dominguez et al. 2003) has shown the presence of 37 conserved short linkage blocks, strong

evidence for microsynteny, but much genome rearrangement on a larger scale. Both of these findings have been corroborated by comparison of genomic sequence between *Arabidopsis* and peach (Georgi et al. 2003). Clearly, this highly saturated reference map has proven its worth in studies of structural genomics. Its value in other areas will be discussed shortly.

EST Database and the Genome Database for Rosaceae (GDR)

Expressed sequence tags, or ESTs, were originally proposed as a way to characterize cDNA libraries from specific tissues, and generate large numbers of candidate genes (Putney et al. 1983, Adams et al. 1991). ESTs are developed by creating cDNA libraries of various tissues, and performing single-pass sequencing of a large number of clones; the entire cDNA sequence is not generally obtained, except for very short transcripts. These libraries are normally directional, allowing for consistent 5'-end sequencing in order to focus on translated regions of the gene, or consistent 3'-end sequencing designed to capture the 3'-UTR (untranslated region) that can be used to distinguish members of multigene families.[2]

The Genome Database for Rosaceae (GDR) is an online resource of Rosaceae sequence and mapping data, managed from Clemson University (Jung et al. 2004, Main et al. 2004). In addition to the current T X E reference map and other Rosaceae linkage maps, all publicly available (from NCBI GenBank) EST sequence data for members of the Rosaceae family can be accessed here. Other online Rosaceae databases exist, such as ESTree for peach (www.itb.cnr.it/ESTree), ESTAP for strawberry (http://staff.vbi.vt.edu/estap/intro.shtml), and the Apple EST Project (http://titan.biotec.uiuc.edu/apple/), but they are species-specific; the GDR represents a

[2] Although mRNAs do contain a 5' UTR, cDNA clones are rarely full-length. In this case, 5'-end sequencing does focus on the translated portion of the transcript.

unified, family-wide resource. Users can perform BLAST homology searches against all or any of these ESTs and search many of them by their annotation, which can include putative function, presence of SSRs, contig position, and T X E map location (www.mainlab.clemson.edu/gdr/). After apple, which currently (April 2005) numbers 185,093 ESTs, the genus *Prunus* contains the second-largest number of ESTs identified so far with 40,851, of which 21,937 come from peach. Nearly 10,000 of these ESTs have been isolated from peach mesocarp tissue by the Abbott laboratory at Clemson. In addition to cDNA library creation, clone isolation, and sequencing, the Abbott group has also performed extensive quality analysis, alignment, and homology searches of these ESTs, resulting in the generation of 3,842-member set of putative unigenes, the first publicly available unigene set for the Rosaceae.

Homology searches of the GenBank nr protein database revealed that a surprising 24.3 percent of these unigenes are novel, with unknown function. Many of these genes may be peach- or at least fruit-specific (Horn et al. 2005), a prime example of the value of economically important model organisms. Work is currently underway to expand the unigene set with genes identified from peach stem and root tissues. Furthermore, as a result of the extremely high sequence conservation of coding regions amongst Rosaceae family members (D. Main, personal communication), a family-wide unigene set is being compiled. ESTs used for the generation of this set are derived from almond, apple, apricot, peach, pear, rose, and strawberry as these are the species currently represented in the GDR and other public databases.

Physical/Transcript Mapping

One of the ultimate goals of genomics is to obtain the entire DNA sequence of the organism being studied. This is not practical without the development of a physical map, an ordered set of large-insert genomic DNA clones, that can then be subcloned and sequenced (whole-genome shotgun sequencing is an alternative sequencing strategy that can theoretically bypass the physical mapping step [Weber and Meyers 1997], but it has been shown to result in difficulty assembling large duplicated segments of repetitive DNA [She et al. 2004]). Generation of a physical map requires two components; a saturated linkage map with many markers that can be used to anchor the physical map, and a large-insert genomic DNA library that 'catalogs' the entire genome of interest, and from which clones comprising the physical map can be selected. A physical map also allows for candidate genes (identified from ESTs) to be more quickly localized to specific chromosomal regions, rather than relying on linkage mapping which requires that polymorphisms of ESTs be present in the population used to construct the map. Due to the existence of a highly saturated general *Prunus* linkage map and a small genome, peach has become the focus of library generation, physical and transcript mapping, and sequencing for the Rosaceae.

Several large-insert genomic libraries (BAC libraries) have been created for peach (Wang et al. 2001, Georgi et al. 2002, Georgi et al. unpublished). The library generated by Georgi et al. (2002) is currently providing the majority of data used to develop the physical map for peach. This BAC library was developed by partial HindIII digestion of high-molecular-weight DNA from the nematode-resistant peach rootstock cultivar 'Nemared', and contains 44,160 clones with an average insert size of approximately 60 kb; this represents an 8 to 9-fold coverage of the haploid peach genome. A second BAC library (Georgi et al.

unpublished) of Sau3AI-digested DNA from the important rootstock cultivar ‘Lovell’, contains approximately 35,000 clones with an average insert size of 80 kb, giving a theoretical 12-fold coverage. This library has been used to complement the ‘Nemared’ library in physical map construction by filling in gaps. Another useful feature of this library is that the ‘Lovell’ clone used is a doubled haploid; it is homozygous at all loci, simplifying the process of assembling clones into a physical map.

Construction of the framework of the peach physical map, as well as initial mapping of ESTs, has been described by Horn et al. (2005). To begin the process of anchoring the physical map to the genetic map, a total of 141 probes representing 153 markers from the T X E reference map were used to probe the ‘Nemared’ BAC library. All genetically mapped probes were successfully hybridized, allowing 679 BAC clones to be anchored to the reference linkage map. In addition to the mapped genomic probes, over 1300 peach ESTs were hybridized to the ‘Nemared’ library. These ESTs identified an additional 4,983 BAC clones. Of these ESTs, 147 (11.2%) were immediately localized to the map, due to their hybridization with one or more of the BAC clones already anchored. As these ESTs are now mapped, they can serve as candidate genes for any traits, including those with polygenic contributions (QTL) that map closely to the original markers used to detect the trait/QTL.

At the time of this writing, over 250 genetically mapped markers and more than 2,700 ESTs had been hybridized to the ‘Nemared’ library (data not shown). Construction of the physical map and localization of ESTs is ongoing; the current status of the project can be viewed on the GDR website. Current results with respect to ESTs are similar to the initial work reported by Horn et al. (2005); approximately 13% of tested ESTs hybridize to genetically anchored BACs. Of the ESTs used so far to probe the ‘Nemared’ library,

approximately 15% have not generated any hits. A number of these 'no-hit' ESTs have already been used to probe the newer, deeper-coverage 'Lovell' library (Georgi et al., unpublished), where 60% gave positive hybridization signals. In addition to genetically anchored markers and ESTs, resistance gene analogues (RGAs) and resistance associated genes (RAGs) are being used as probes. These can serve as candidate genes for disease resistance loci, and one RAG has already been localized to a genetically anchored contig containing a marker for plum pox virus (PPV) resistance (V. Decroocq, unpublished results).

Genetically unanchored BAC clones are being organized into contigs via BAC fingerprinting (Hoskins et al. 2000). Briefly, DNA from random BAC clones or those identified by EST hybridization are digested with restriction enzymes and separated by gel electrophoresis, and their banding profiles are compared to determine the degree of overlap between clones using FingerPrinted Contigs (FPC) software (Soderlund et al. 2000). In all, 15,000 BAC clones from both libraries have been fingerprinted and used to generate contigs for the physical map, which can also be viewed in an interactive display at the GDR website. More markers from the T X E and other *Prunus* maps are also being used as BAC library probes to genetically anchor more clones. The physical map should be complete (all euchromatic regions represented) within a year (spring 2006, A. Abbott, personal communication).

The *evergrowing* locus

One particularly important example of the usefulness of peach as a model organism for Rosaceae is the research surrounding the *evergrowing* (*evg*) locus. Originally identified in southern Mexico (Diaz 1974), the phenotype is characterized by a failure of terminal buds

to enter winter dormancy, and an elimination of the chilling requirement. This is an advantage in warm climates, allowing the trees to continue to grow as well as produce fruit; indeed two crops a year are realized in Mexico (Wang et al. 2002b). Genetically, *evg* behaves as a single recessive gene (Rodriguez et al. 1994). This mutation and its qualitative nature, make it an ideal candidate for the study of the genetics of dormancy induction, an important characteristic of most temperate-climate perennial plants (Bielenberg et al. 2004).

The segregating population currently under study for *evg* is an F2 derived from a cross between the cultivar 'Empress' (wild-type dormant) and the selection P.I. 442380 (non-dormant) (Werner and Okie 1998). Wang et al. (2002b) used a combination of bulk segregant analysis (BSA, Vos et al. 1995) and AFLP markers to generate a saturated linkage map of the *evg* region in this population. One STS marker converted from AFLP marker EAT/MCAC mapped within 1 cM of *evg*. This marker was subsequently used to generate a physical map of the *evg* locus, based on the 'Nemared' BAC library (Wang et al. 2002a). Clone 018F12 from this library was shown to contain the STS marker, and to overlap with a second clone, 109L12. SSRs are quite common in peach (Georgi et al. 2003), and one, pchgms29, was found in clone 109L12 that maps 2.9 cM from EAT/MCAC, and on the opposite side of *evg* (Wang 2002). End-sequencing of 018F12 revealed an ORF with homology to MADS-box transcription factors (Georgi et al. 2003).

This ORF was then used as a probe in Southern blots of BACs comprising the contig spanning *evg*, as well as wild-type and non-dormant peach trees (Bielenberg et al. 2004). Analysis of these Southern blots showed that there are six MADS-box-homologous genes at or near the *evg* locus, and that a deletion in non-dormant genotypes resulted in the loss of

four of these genes. These four genes (more accurately ORFs) represent the best candidate genes to date for the *evg* locus (Bielenberg et al. 2004).

The location of the *evg* locus has been identified. Proof or disproof of one or more of these MADS-box ORFs would require transformation of *evg* mutants with each of the candidates, and a corresponding rescue to a dormant phenotype. Routine transformation and regeneration has been developed for plum (Gonzalez et al. 2003, Callahan 2004), but has been problematic for peach (Scorza et al. 1989). Recently, Pérez-Clemente et al. (2004) reported reliable whole-plant regeneration of *Agrobacterium*-transformed embryo-derived peach tissues. Though this method relies on sexually derived tissue, and therefore cannot be used to enhance existing cultivars, the development does represent a significant milestone in peach genomics as we now have the tools to perform phenotype conversions/complementation to confirm candidate genes. In addition, RNA interference (RNAi), which is used to inhibit expression of genes of interest, has been reported in whole peach trees (Horn et al. 2004). As these methods are more widely disseminated, we should see their application to genomics studies of peach, including the identification of gene(s) responsible for the *evg* mutation.

Summary and Conclusion

The development of peach as a model structural genomic species for the Rosaceae has become a worldwide effort, with 20 laboratories participating (Abbott et al. 2002). The collaboration of these groups has accelerated the progress of research, both by pooling of resources and data and by generating a stronger, more united voice for the request of research funds. A saturated *Prunus* reference map has been developed and utilized for localization of

a number of important traits, and comparative mapping within and outside the genus. The wealth of sequence data in the GDR is being used to construct a 60,000-gene microarray to test the feasibility of a family-wide array for functional genomics studies. The reference map and peach BAC libraries have been used to construct a genetically anchored physical map for peach, to which a number of ESTs have been localized. A method for stable transformation and regeneration of peach, hitherto unavailable, has now been developed, which will greatly enhance the study of functional genomics in peach. Research funding for genomics studies in the Rosaceae has now been given priority by the USDA's National Research Initiative (NRI) for 2004-2005 (www.mainlab.clemson.edu/gdr/community/funding/index.shtml), and funding has been secured for completion of the peach physical map (Abbott et al. 2005). International and U.S. Rosaceae Genomics, Genetics and Breeding National Steering Committees have been formed to help focus the research in this family, and prevent duplications in effort. As a result, a three-genera (*Prunus*, *Malus*, and *Fragaria*) research system for Rosaceae has been proposed, with the sequencing of the entire peach genome forming the structural genomics 'pillar' of the research effort (RosaGEC 2005). A hybrid approach modeled after the one used to sequence the mouse genome (Mouse Genome Sequencing Consortium 2002) has been suggested for peach (A. Abbott, R. McCombie, and B. Sosinski, personal communication). This would involve sequencing the completed physical map at low (~75%) coverage, followed by whole genome shotgun (WGS) sequencing at approximately 8X coverage to fill in the small gaps that remain. This hybrid approach is more efficient than pure WGS, and is less expensive than the strict clone-by-clone approach. Furthermore, this method facilitates collaboration, as individual laboratories can contribute WGS data as their funds allow. Collaboration will also increase the peach

SNP (single nucleotide polymorphism) database as different cultivars are used by laboratories throughout the world.

Peach is a prime candidate for sequencing amongst the Rosaceae members because of its small genome, the fact that it is a tree (it will be only the second tree genome to be sequenced, after black poplar (DOE-JGI 2004), and its close structural genomic relationship to a large number of other important species (the genus *Prunus*). The full genome sequence of peach will provide information on upstream regions of genes (promoters and enhancers) that are important for gene regulation, aid in identifying genes not discovered through EST sequencing projects, and provide a 'reference' genome for comparative genomics amongst other members of the Rosaceae (RosaGEC 2004). The combination of the intrinsic advantages of peach as a model organism, the advent and application of high-throughput technologies for genetic and genomic analyses, and the collaboration of a large number of talented researchers is already generating a wealth of information, and will continue to provide valuable insights on an important family of flowering plants.

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Tables and Figures

Table 1. Linkage maps published for peach and other *Prunus* species. Historical and current maps are listed for peach, while only the more recently published maps are listed for the other species.

Reference	**Marker type**	**Number of markers**	**Number of linkage groups**	**Map length**
Bailey and French 1940, 1949	Morphological	4	1	55 or 60 cM
Monet et al. 1985	Morphological	2	1	30 cM
Monet and Gibault 1991	Morphological Isozyme	2	1	4 cM
Belthoff et al. 1993	RFLP	35	5	N/A
Chaparro et al. 1994	RAPD Morphological	83	15	396 cM
Dirlewanger and Bodo 1994	RAPD Morphological	49	8	350 cM
Rajapakse et al. 1995	RFLP RAPD Morphological	47	8	332 cM
Abbott et al. 1998[3]	RFLP RAPD AFLP SSR	209	8	450 cM
Dirlewanger et al. 1998	RFLP RAPD AFLP Morphological Isozyme	249	11	712 cM
Quarta et al. 1998	RFLP RAPD Morphological	36	8	257 cM
Joobeur et al. 1998[4]	**RFLP Isozyme**	**246**	**8**	**491 cM**

[3] Combined data from three distinct populations.
[4] Interspecific (almond X peach) cross.

Table 1-- continued

Lu et al. 1998	AFLP	153	15	1297 cM
Dettori et al. 2001	RFLP RAPD SSR Morphological Isozyme	109	10	521 cM
Jáuregui et al. 2001[3]	RFLP Isozyme	51	7	439 cM
Yamamoto et al. 2001	RFLP RAPD SSR ISSR AFLP Morphological	92	9	1020 cM
Bliss et al. 2002[3]	RFLP RAPD SSR CAP Morphological Isozyme	161	8	1144 cM
Etienne et al. 2002	RFLP RAPD SSR IMA Morphological Isozyme	71	8	564 cM
Foulongne et al. 2003a[5]	RFLP SSR AFLP	153	8	874 cM
Joobeur et al. 2000[6]	RFLP RAPD SSR Isozyme	126-- ♀ parent 99-- ♂parent	8	415 cM-- ♀ 416 cM-- ♂
Lambert et al. 2004[7]	RFLP SSR	110-- ♀ parent 141-- ♂parent	8	538 cM-- ♀ 699 cM-- ♂
Dirlewanger et al. 2002b[8]	SSR AFLP	69	10-- ♀ parent 9-- ♂parent	345 cM-- ♀ 218 cM-- ♂
Canli 2004[9]	RFLP SSR	161	19	442.4 cM

[5] F_2 population of peach X *P. davidiana* cross
[6] Almond
[7] Apricot
[8] Sweet cherry
[9] Sour cherry

Table 1-- continued

Dirlewanger et al. 2004b[10]	SSR SCAR	94	8	653.8 cM

[10] Myrobalan plum (*P. cerasifera*)

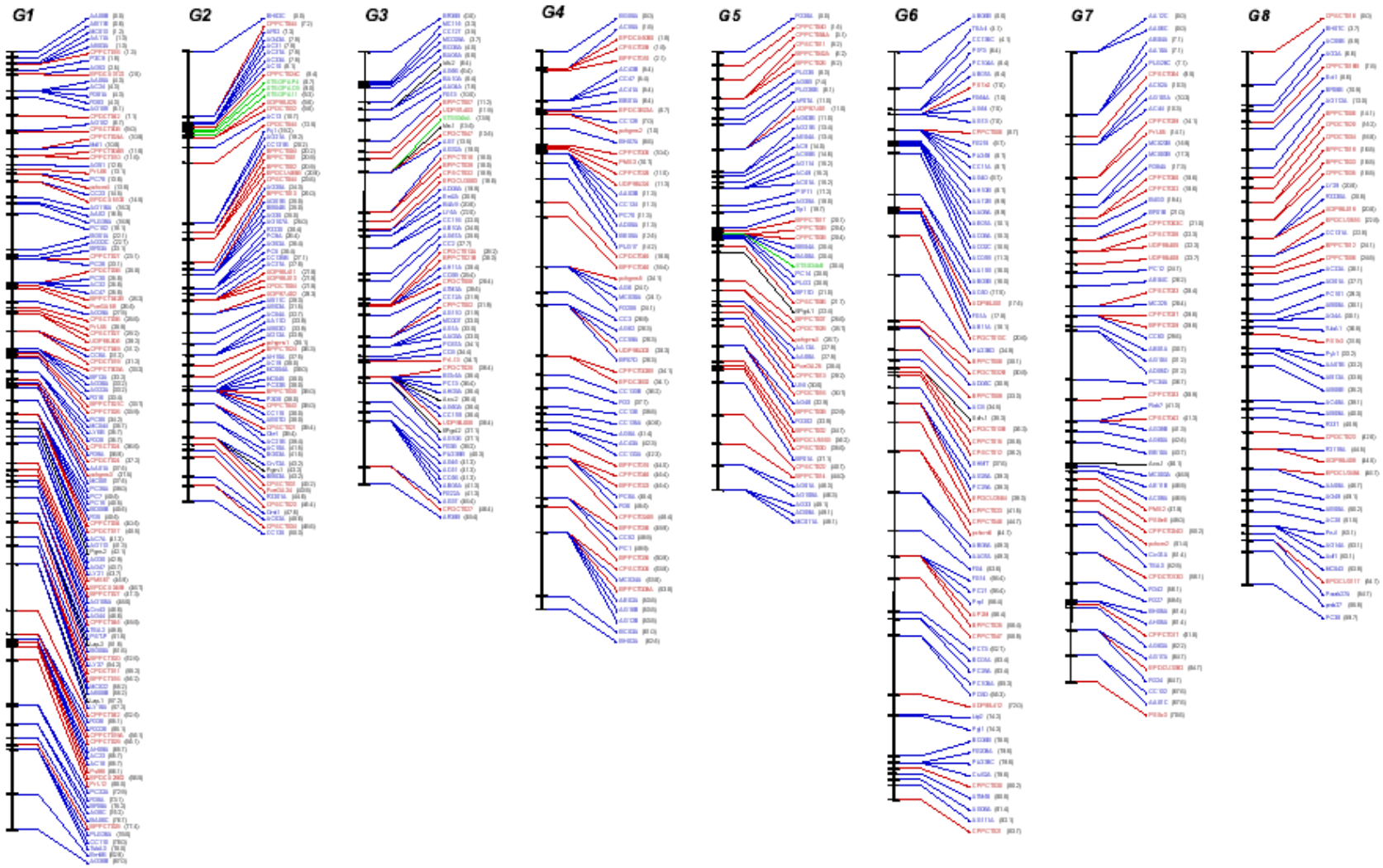

Figure 1. The current *Prunus* reference map, adapted from Dirlewanger et al. 2004a. Click here for a high-resolution version.

Table 2. Description of major genes (21) and QTL (28) affecting morphological or agronomic characters in peach that can be placed on the *Prunus* reference map.

Characters	**L.G.** [(1)]	**Symbol**[(2)]	**Populations**	**References**
Flesh color (white / yellow)	G1	*Y*	'Padre' x '54P455'	Warburton et al. (1996); Bliss et al. (2002)
evergrowing	G1	*evg*	'Empress dwarf' x PI442380	Wang et al. (2002)
Internode length	G1	QTL	(*P. ferganensis* x 'IF310828')BC1	Verde et al. (2002)
Powdery mildew resistance	G1	QTL	'Summergrand' x P1908	Foulongne et al. (2003b)
Flower color	G1	*B*	'Garfi' x 'Nemared'	Jáuregui (1998)
Root-knot nematode resistance	G2	*Mi*[(3)]	'P.2175' x 'GN22' 'Akame' x 'Juseitou', 'Lowell' x 'Nemared','Garfi' x 'Nemared', 'Padre' x '54P455'	Claverie et al. (2004);Yamamoto et al. (2001); Lu et al. (1998); Bliss et al. (2002); Jáuregui (1998)
Ripening time, fruit skin color, Soluble-solids content	G2	QTL	(*P. ferganensis* x 'IF310828') BC1	Verde et al. (2002)
Double flower	G2	*Dl*	'NC174RL' x 'PI'	Chaparro et al. (1994)
Broomy (or pillar) growth habit	G2	*Br*	Various progenies	Scorza et al. (2002)
Flesh color around the stone	G3	*Cs*	'Akame' x 'Juseitou'	Yamamoto et al. (2001)
Anther color (yellow/anthocyanic)	G3	*Ag*	'Texas' x 'Earligold'	Joobeur (1998)
Leaf curl resistance	G3	QTL	'Summergrand' x P1908	Viruel et al. (1998)
Fruit weight, fruit diameter, glucose content	G3	QTL	'Suncrest' x 'Bailey'	Abbott et al. (1998)
Polycarpel	G3	*Pcp*	'Padre' x '54P455'	Bliss et al. (2002)
Flower color	G3	*Fc*	'Akame' x 'Juseitou'	Yamamoto et al. (2001)
Blooming time, ripening time, fruit development period	G4	QTL	'Ferjalou Jalousia®' x 'Fantasia'; (*P. ferganensis* x 'IF310828') BC1	Etienne et al. (2002); Verde et al. (2002)
Soluble-solids content, fructose, glucose	G4	QTL	'Ferjalou Jalousia®' x 'Fantasia'	Etienne et al. (2002)
Flesh adhesion (clingstone / freestone)	G4	*F*	(*P. ferganensis* x 'IF310828')BC1; 'Akame' x 'Juseitou'	Verde et al. (2002); Dettori et al. (2001); Yamamoto et al. (2001)

Table 2--continued

Non-acid fruit	G5	*D*	'Ferjalou Jalousia®' x 'Fantasia'	Dirlewanger et al. (1998, 1999); Etienne et al. (2002)
Sucrose, malate, titratable acidity, pH, sucrose	G5	QTL	'Ferjalou Jalousia®' x 'Fantasia'	Etienne et al. (2002)
Skin hairiness (nectarine / peach)	G5	*G*	'Ferjalou Jalousia®' x 'Fantasia'; 'Padre' x '54P455'	Dirlewanger et al. (1998, 1999); Bliss et al. (2002)
Kernel taste (bitter / sweet)	G5	*Sk*	'Padre' x '54P455'	Bliss et al. (2002)
Ripening time, fruit skin color, soluble-solids content	G6	QTL	(*P. ferganensis* x 'IF310828') BC1	Verde et al. (2002)
Plant height (normal / dwarf)	G6	*Dw*	'Akame' x 'Juseitou'	Yamamoto et al. (2001)
Leaf shape (narrow / wide)	G6	*Nl*	'Akame' x 'Juseitou'	Yamamoto et al. (2001)
Male sterility	G6	*Ps*	'Ferjalou Jalousia®' x 'Fantasia'	Dirlewanger et al. (1998)
Powdery mildew resistance	G6	QTL	'Summergrand' x P1908	Foulongne et al. (2003b)
Leaf curl resistance	G6	QTL	'Summergrand' x P1908	Viruel et al. (1998)
Fruit shape (flat / round)	G6	*S**	'Ferjalou Jalousia®' x 'Fantasia'	Dirlewanger et al. (1998, 1999)
Leaf color (red / yellow)	G6-G8	*Gr*	'Garfi' x 'Nemared'; 'Akame' x 'Juseitou'	Jáuregui (1998); Yamamoto et al. (2001)
Fruit skin color	G6-G8	*Sc*	'Akame' x 'Juseitou'	Yamamoto et al. (2001)
Leaf gland (reniform / globose / eglandular)	G7	*E*	(*P. ferganensis* x 'IF310828') BC1	Dettori et al. (2001)
Resistance to mildew	G7	QTL	(*P. ferganensis* x 'IF310828') BC1	Verde et al. (2002)
Powdery mildew resistance	G8	QTL	'Summergrand' x P1908	Foulongne et al. (2003b)
Quinase	G8	QTL	'Ferjalou Jalousia®' x 'Fantasia'	Etienne et al. (2002)

(1) LG = Linkage group; G6-G8 genes located close to the translocation breakpoint between these two linkage groups.

(2) QTL are included if they have been consistently found (at least in two independent measurements) in the indicated populations.

(3) One or two genes of nematode resistance with different notations and one QTL have been described in this linkage group.

Chapter Two

Physical mapping of fruit-derived ESTs from peach, a model for *Prunus* and Rosaceae structural genomics

Abstract

Peach (*Prunus persica* [L.] Batsch) has been proposed as a model organism for structural genomics in the family Rosaceae. Several resources have been or are currently being developed to study the structural genomics of the species. A highly saturated genetic linkage map has been developed from an interspecific 'Texas' almond X 'Earligold' peach cross (T X E), and two peach BAC libraries from the cultivars 'Nemared' and 'Lovell' have been generated for the creation of a physical mapping resource. A framework map has been generated by the hybridization of markers from the T X E and other *Prunus* maps to the 'Nemared' library. In addition, peach and almond unigenes are being hybridized to the library in order to generate an anchored transcript map, identify contigs for overall map construction, and provide information on genome organization. A subgroup of the *Prunus* unigene set comprising 942 ESTs derived from peach fruit mesocarp was hybridized to the 44,150-clone 'Nemared' library. Eight hundred ninety-nine of these ESTs successfully hybridized to the library, identifying 3475 BACs, of which 2725 were unique. Seventy-six of these ESTs are now anchored to the reference map by hybridization to genetically anchored BAC clones or contigs. Twenty-six of these ESTs mapped near loci for important agronomic traits, including flesh acidity, fruit skin pubescence, and almond shell hardness. Unanchored ESTs generated contigs of 2 to 44 clones, which are being fingerprinted and integrated into the growing peach physical map in preparation for sequencing of the peach genome.

Introduction

Peach (*Prunus persica* [L.] Batsch) has been proposed as a model organism for structural genomics in the family Rosaceae (Abbott et al. 2002, Horn et al. 2005, RosaGEC 2005). Peach has a number of characteristics that make it well-suited for such research. It is a self-pollinating diploid (2n = 16) with a small genome (~300 Mb, approximately twice the size of *Arabidopsis thaliana*), a short juvenility period of 2-3 years, and is well-characterized genetically, with a significant number of single-gene traits documented in the literature (Scorza and Sherman 1996 2004).

Several resources have been or are currently being developed to study the structural genomics of peach. A highly saturated genetic linkage map has been developed from an interspecific 'Texas' almond X 'Earligold' peach cross (Joobeur et al. 1998, Dirlewanger et al. 2004a). The T X E map, as it is called, has already proven its value by anchoring other genetic maps developed for *Prunus*, and in comparative mapping within and outside the genus. In addition, two peach BAC libraries from the cultivars 'Nemared' (Georgi et al. 2002) and 'Lovell' (Georgi et al., unpublished) have been generated for the creation of a physical mapping resource. Horn et al. (2005) began the process of generating the physical map, using markers from the T X E reference map as probes of the 'Nemared' library. This created an initial group of genetically anchored BAC clones, which is expanding as more markers from the T X E and other *Prunus* maps are used to probe the BAC libraries.

In addition to anchoring the physical map with genetic markers, it is desirable to localize actual genes on the map. Direct hybridization of expressed sequence tags (ESTs) to a BAC library is an efficient method of mapping genes, if the marker saturation of the genetic map is high. Any EST that hybridizes to the same BAC clone or contig as a mapped

marker can be considered itself mapped (Wu et al. 2002). These anchored ESTs can then serve as candidate genes for any traits or QTL that map to the same region. For example, Nothwang et al. (1997) used a combination of PCR screening and hybridization to anchor 58 ESTs to the human physical map, in a region flanked by markers for two genes implicated in mental retardation. Even ESTs that are not immediately genetically anchored can generate contigs useful for physical map construction. A final benefit of EST mapping is the information it provides on the gene space of the organism under study; clustering of ESTs provides direct evidence of gene-rich regions.

Initial results of an international effort to physically map 3,000 unique peach and almond ESTs (the *Prunus* unigene set) were published by Horn et al. (2005). Here I present my contribution to this effort, the hybridization of nearly 900 peach ESTs to the 'Nemared' peach BAC library.

Materials and Methods

Membrane processing and EST probe labeling

The 'Nemared' peach BAC library, consisting of 44,160 clones with an average insert size of 60 kb (Georgi et al.2002), and 960 ESTs from the peach unigene set (Horn et al. 2005), were supplied by the A. Abbott laboratory at Clemson University and the Clemson University Genome Institute (CUGI). The BAC library was provided as 115 384-well plates, and the ESTs were supplied as bacterial cultures in 96-well format (10 plates). The BAC library was arrayed on three 22 X 22 cm nylon membranes in a 4 X 4 gridding pattern using a Genetix Q-bot (Figures 1 and 2). Membranes were incubated overnight at 37° C on LB-agar trays supplemented with 12.5 μg/mL of chloramphenicol. The membranes were

incubated for 7 minutes on sheets of Whatman 3M paper saturated with 0.4M NaOH, 0.5M NaCL, to lyse the bacterial cells and denature the DNA. Membranes were then neutralized by incubating for 7 additional minutes on paper saturated with 0.5M NaCl, 0.1M Tris-Cl pH 8.0. DNA was fixed to the membranes by either the alkaline lysis method (air-dried membranes incubated for 20 minutes on paper saturated with 0.4N NaOH, followed by neutralization for 7 minutes on paper saturated with 4X SSC buffer), or by UV crosslinking (125 mJ). ESTs were amplified directly from bacterial cultures, bypassing the isolation of plasmid DNA. This method was further streamlined by using a 96-pin manual replicator dipped in each plate to add template to the PCR reactions, eliminating an extra pipetting step. PCR reactions were performed in 25 μL reactions containing 1X PCR buffer with 1.5mM $MgCl_2$, 2mM (each) dNTPs, 0.4μM each of T3 (5'-ATTAACCCTCACTAAAG-3') and T7 (5'-AATACGACTCACTATAG-3') primers, and 1 unit *Taq* polymerase. Reaction volume was calculated without regard to the addition of bacterial template via the replicator, but this did not affect results (P. McCord, unpublished results). Reaction conditions were as follows: Initial lysis and denaturation for 4 minutes at 94˚C, followed by 25-30 cycles of 30 seconds at 94˚ C, 30 seconds at 55˚ C, and 2 minutes at 72˚ C, with a final extension of 7 minutes at 72˚ C.

Probes were labeled initially via the random hexamer/Klenow fragment method used by Horn et al. (2005, volume increased to 50 μL), but it was later discovered that labeling via PCR resulted in lower background signal, even when unincorporated radionucleotides were not removed. The newer method also uses much less template DNA (0.5-10 vs. 100-150 ng), and uses less expensive enzyme and primers. PCR labeling was performed in an Eppendorf Mastercycler in 20 μL reactions containing 1X PCR buffer with 1.5mM $MgCl_2$, 2mM dATP,

dGTP, and dTTP, 0.1mM dCTP, 1µL (10µCi) α^{32}P-dCTP, 0.4µM each of T3 and T7 primers, 0.5-15 ng DNA from the amplification reaction, and 1 unit *Taq* polymerase. Cycling conditions were similar to those used for amplification, with the exception that the initial denaturation time was reduced to 2 minutes, 30 seconds. Unincorporated nucleotides were removed by centrifugation through mini-columns of Biogel P30 (BioRad) or Sephadex G-50 (Sigma). This procedure was not always necessary, particularly with BAC membranes that had been used repeatedly, and the utilization of PCR labeling. Reaction cleanup did have the advantage of indicating the success of the labeling reaction (which was generally robust with either method).

Probe hybridization and data collection

To eliminate ESTs with repetitive sequences (these would have resulted in spurious hybridization to large numbers of BAC clones), all 960 ESTs were screened via colony hybridization, using doubled haploid 'Lovell' genomic DNA as a probe. Remaining ESTs were hybridized in pools of 10-18 probes which increased the longevity of the BAC library membranes which are expensive to produce, and increased the rate at which probes could be assigned to BACs. Hybridization and washing were performed at 65˚ C in a rotating hybridization oven. Following prehybridization (minimum 2 hours for new membranes, 1 hour for used ones), membranes were hybridized with probes overnight in 25-50 mL (depending upon numbers of probes and age of membranes) of buffer (0.25M sodium phosphate buffer pH 7.2, 1mM EDTA pH 8.0, 7% SDS). Membranes were washed twice in 1X SSC, 0.1% SDS for 20-60 minutes, and once or twice (depending on background signal) in 0.5X SSC, 0.1% SDS for 15-60 minutes. Membranes were blotted dry, wrapped in plastic

wrap, and exposed at room temperature to Kodak storage phosphor screens for 3 to 60 hours, depending on signal strength. Before reuse, membranes were stored until signal strength had significantly decayed (approximately 1 month), or chemically stripped by two 10-minute incubations in 100mM NaOH, 10mM EDTA pH 8.0, 0.1% SDS, followed by two 10-minute incubations in 5X SSC buffer. Images (Figure 3) were collected using an FX phosphoimager and Quantity One software (BioRad), enhanced if necessary for readability with either Quantity One or Adobe PhotoShop, and scored using Incogen's High Density Filter Reader software, version 2.0. This software is capable of detecting hits (hybridization signals) and determining their clone addresses. Though the software proved difficult to use for hit detection (high background signals were often identified as hits), the automatic address identification greatly speeded the process of scoring images.

To determine which probes from the multiplexed hybridizations corresponded to which hits, all positive BAC clones identified in a given hybridization were picked from frozen cultures and grown overnight at 37˚ C in LB supplemented with 12.5 μg/mL chloramphenicol. A 96-pin manual replicator was used to stamp multiple copies of this sub-library onto nylon membranes. One copy was made for each probe used in the original hybridization. Following overnight incubation and membrane processing, these membranes were prehybridized as described above before addition of probe(s) (half the initial labeling reaction was saved for this second hybridization). Following overnight hybridization, membranes were washed twice in 1X SSC, 0.1% SDS for 10 to 40 minutes, wrapped in plastic wrap, and exposed and imaged as for the larger BAC membranes (Figure 4). Probes that did not hybridize were tested again to confirm the negative result. Hits for each EST probe were scored manually and data were entered into an Excel spreadsheet, which was then

submitted to the Abbott laboratory at Clemson University. There, genetically anchored ESTs are identified, contigs are fingerprinted, and the results incorporated into the Genome Database for Rosaceae (www.mainlab.clemson.edu/gdr, Horn et al. 2005).

Results and Discussion

Of the 960 ESTs originally received, 15 were identified in the initial screen for repetitive sequences and were not used. Three clones failed to grow and/or amplify, leaving 942 ESTs to be used as probes (Appendix). One additional EST was subsequently identified during the course of the experiment as containing repetitive sequences and was omitted from the hybridization dataset, and 42 ESTs (4.5%) did not hybridize to any clones in the library. The percentage of non-hybridizing ESTs is significantly lower than that reported by other researchers using the same BAC library (15%, Abbott et al. 2005); this is likely the result of testing each 'no-hit' EST twice before considering it to be non-hybridizing. The remaining 899 ESTs detected a total of 3475 BACs, an average of 3.87 BAC clones per EST probe (Table 1). This is in excellent agreement with the overall estimate of 3.8 clones/probe by Horn et al. (2005). The range of clones identified by each probe was 1 to 44, (Figure 5) similar to that reported by Horn et al. (2005).

Clustering of ESTs was evident from the raw hybridization results. Of the 3475 BAC clones identified, 2725 were unique (Table 1). The ten BAC clones that hybridized to the most ESTs are displayed in Figure 6. As discussed in Horn et al. (2005), clones 028F08 and 082I18, which have an overlap of 7 kb, are rich in ESTs, with 22 and 18, respectively. These clones are directly anchored to the physical map by hybridization with marker AC55, an almond RFLP, and LF11, an RFLP marker from peach. Two other clones, 114I14 and

085P07, are in the same contig as 028F08 and 082I18, and contained 18 and 12 ESTs, respectively. LF11 is located near the bottom of linkage group G6 on the Myrobalan plum X (almond X peach) map (Dirlewanger et al 2004b) and AC55 maps to two separate linkage groups (G4 and G5) on the T X E map, resulting in three possible physical locations for this contig. Both 028F08 and 082I18 were sequenced, and found to be distinct but related segments, likely an ancient duplicated region (R. McCombie and B. Sosinski, personal communication) which has also undergone translocation. The true physical location of each of these BACs and the genes they contain should be resolved as the other clones (085P07 and 114I14) are sequenced, and as the physical map is filled with more contigs.

By using the list of all (not just T X E) genetically anchored BAC clones as a guide, 37 ESTs were genetically anchored by virtue of their hybridization to one or more of these anchored BACs; the majority of these (29) hybridized to 028F08 and/or 082I18. A search was then made of the additional BACs identified by these anchored ESTs that were also identified by other EST probes. These new ESTs were then considered anchored by virtue of their localization to an anchored contig. This procedure was repeated until no additional ESTs were found. This analysis of hybridization data identified an additional 206 putatively anchored ESTs, for a total of 243. According to the GDR, 76 of these ESTs are considered anchored to the T X E and/or other *Prunus* linkage maps (www.mainlab.clemson.edu/gdr) used to anchor the physical map. Twenty-six of these anchored ESTs map closely to important agronomic traits (Appendix), for example non-acid fruit, skin pubescence, and flesh adherence to the stone. Many of these ESTs are anchored to more than one map position, evidence of either multiple copies of the gene, the presence of closely related family members, or possibly discrepancies between the different linkage maps used to anchor the

physical map. In addition, 28 anchored ESTs had no significant homology to any sequence in GenBank, indicating they may be fruit or at least *Prunus*-specific (Horn et al. 2005).

There are several possible reasons for the discrepancy between anchored ESTs listed in the GDR, and ESTs considered anchored according to the hybridization data. The current online database contains EST probes submitted through October of 2004; many ESTs have been hybridized since then, but the data have yet to be made publicly available. In addition, the further analysis of BAC clones by fingerprinting may have rejected some of these putatively anchored ESTs as false positives. These false positives may be the result of the gene being present in multiple copies, hybridization to other members of a gene family, or high background hybridization. In particular, when using the random-priming method for labeling, I found that probes in the same hybridization experiment often co-hybridized to a few (1-4) BACs. These co-hybridization events may be the result of labeling of plasmid vector and/or host cell DNA carried over from the initial template used in the probe amplification reactions. Because most of the DNA fixed to the membranes is also bacterial in origin (either from the BAC vector or host cell), hybridization between EST vector/host DNA and BAC vector/host DNA is much more likely than hybridization between peach EST DNA and its peach genomic DNA target. Enough hybridization between EST vector/host DNA and BAC vector/host DNA could occur that a detectable signal could result. Such a phenomenon would be more apparent when using the random-priming method of labeling, as it requires much more template DNA than the PCR-based method. Finally, as more genetic markers are used to anchor additional BAC clones, the number of genetically anchored ESTs will also increase.

Summary and Conclusion

This work has made an important contribution toward the goal of generating a physical transcript map for peach. Hybridization of 940 ESTs from a portion of the peach unigene set has identified 2725 unique BAC clones. Seventy-six of these ESTs are considered genetically anchored by virtue of their hybridization to genetically anchored BAC clones or contigs. Several of these map closely to important agronomic traits, and can be considered candidate genes for these traits. The remaining ESTs, while as yet unanchored, have generated a number of contigs that are useful for fingerprinting and physical map enrichment. The physical map should be complete (all euchromatic regions represented) within a year (spring 2006, A. Abbott, personal communication). The stage is now set for sequencing of the peach genome. A strategy similar to that used for mouse (Mouse Genome Sequencing Consortium 2002) has been proposed for peach (A. Abbott, R. McCombie, and B. Sosinski, personal communication). This would involve sequencing the completed physical map at low (~75%) coverage, complemented by whole genome shotgun (WGS) sequencing to fill in the small gaps that remain. This hybrid approach is more efficient than pure WGS, and is less expensive than the strict clone-by-clone approach. Furthermore, this method facilitates collaboration, as individual laboratories can contribute WGS data as their funds allow. Collaboration will also increase the peach SNP (single nucleotide polymorphism) database as different cultivars are used by laboratories throughout the world. Peach is a prime candidate for sequencing amongst the Rosaceae members because of its small genome, the fact that it is a tree (it will be only the second tree genome to be sequenced, after black poplar (DOE-JGI 2004), and its close structural genomic relationship to a large number of other important species (the genus *Prunus*).

The full genome sequence of peach will provide information on upstream regions of genes (promoters and enhancers) that are important for gene regulation, aid in identifying genes not discovered through EST sequencing projects, and provide a 'reference' genome for comparative genomics amongst other members of the Rosaceae (RosaGEC 2004). The EST mapping data generated by this research has helped elevate the status of peach research to the point where full genome sequencing can now be considered, which will be the culminating development of peach as a model species for Rosaceae structural genomics.

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Tables and Figures

Table 1. Summary of peach EST hybridization results.

ESTs successfully hybridized	899 (95.4% of non-repetitive ESTs)
Total BAC clones detected	3475
Total unique BAC clones	2725
Genetically anchored ESTs	243 (76 confirmed from GDR)

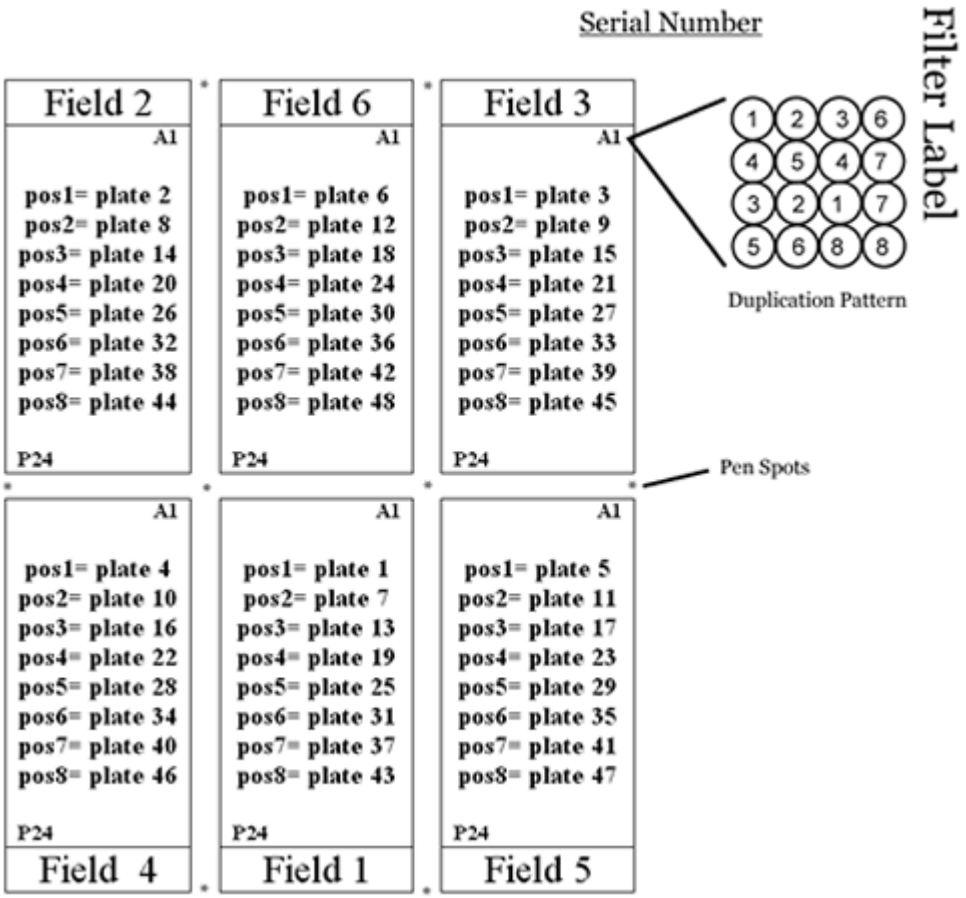

Figure 1. Diagrams outlining spotting pattern of BAC clones (upper right), and the decoding of their location on 6-field arrays (left). Each clone is spotted twice to help eliminate false positives. The first two membranes of the 'Nemared' BAC library, comprising 96 384-well plates, were gridded onto these 6-field arrays. (www.genome.clemson.edu/groups/bac/protocols/fieldchart2.png.)

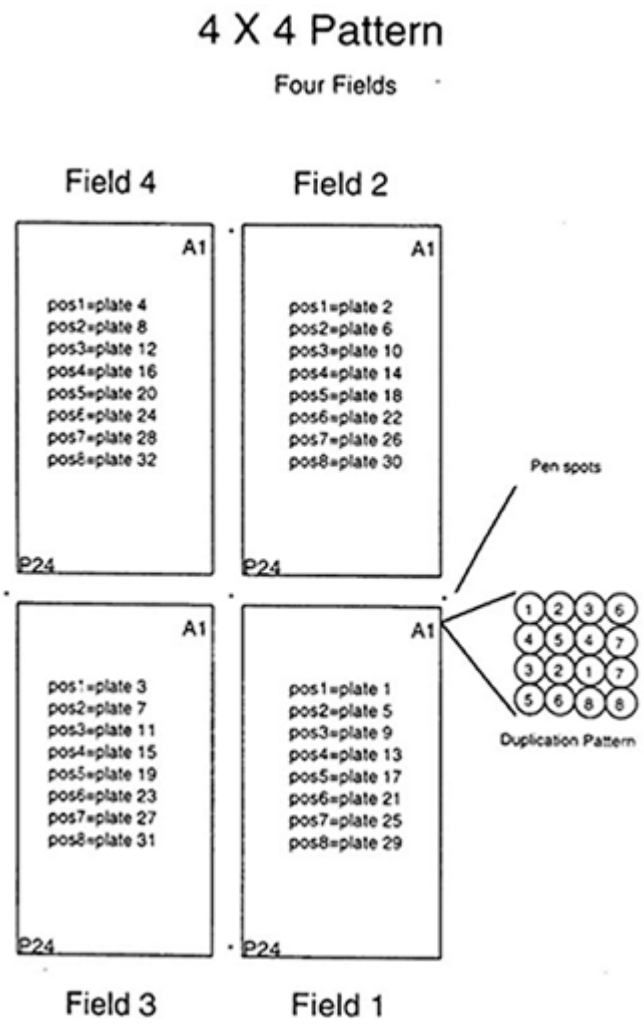

Figure 2. Diagrams outlining spotting pattern (lower right, identical to Figure 1) and the decoding of their location on 4-field arrays. The last membrane, comprising 19 plates of the BAC library, was arrayed in this fashion.
(www.genome.clemson.edu/groups/bac/protocols/4x4field.png.)

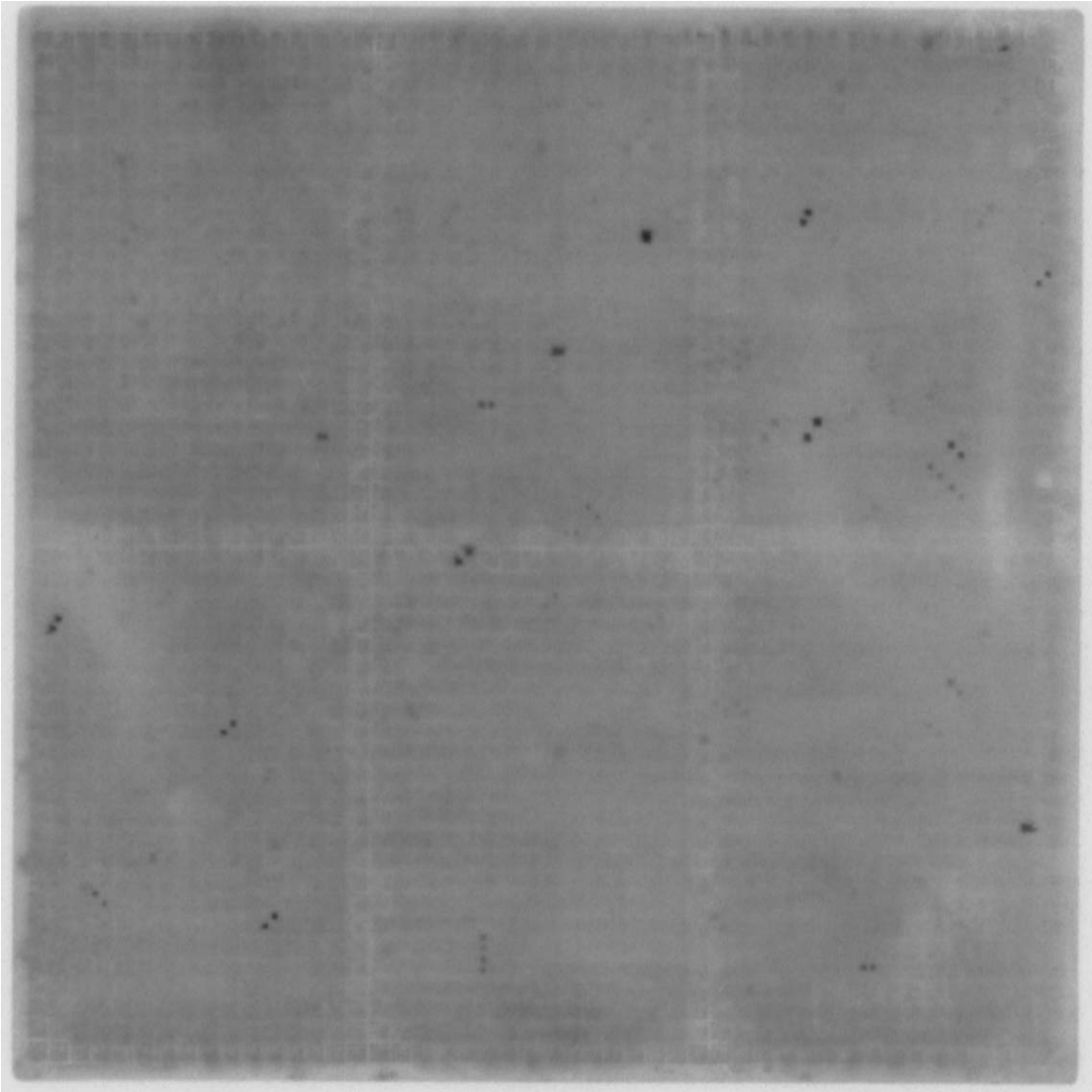

Figure 3. Example of BAC membrane hybridized with 10 EST probes.

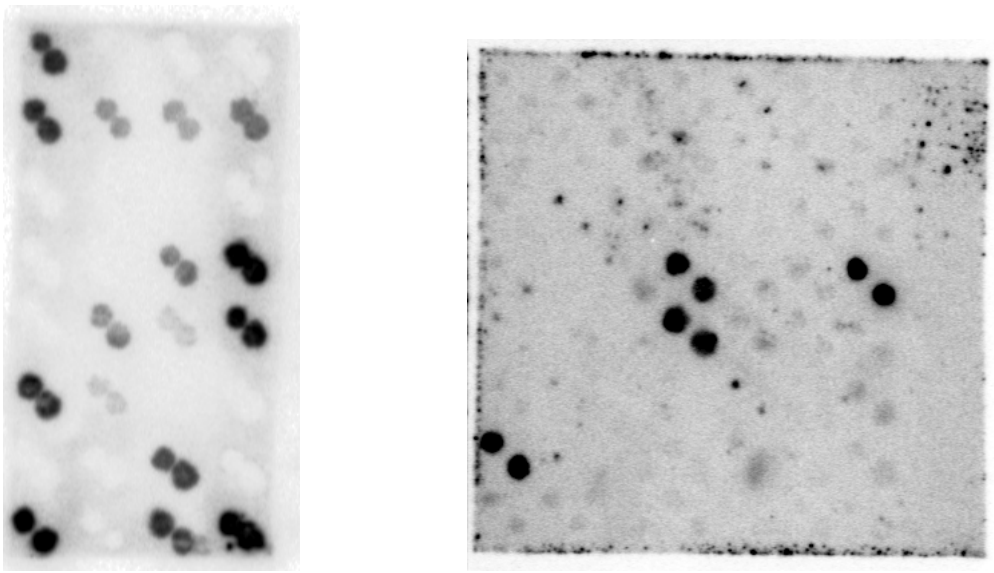

Figure 4. Typical results following hybridization of positive clones from BAC library to individual ESTs.

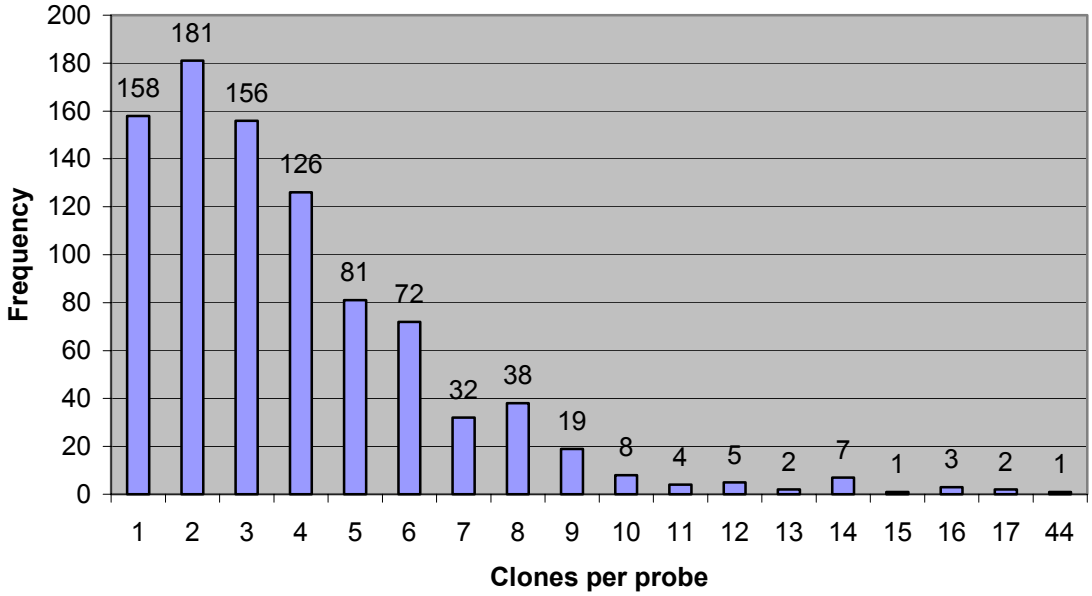

Figure 5. Histogram of the numbers of BAC clones that hybridized to EST probes.

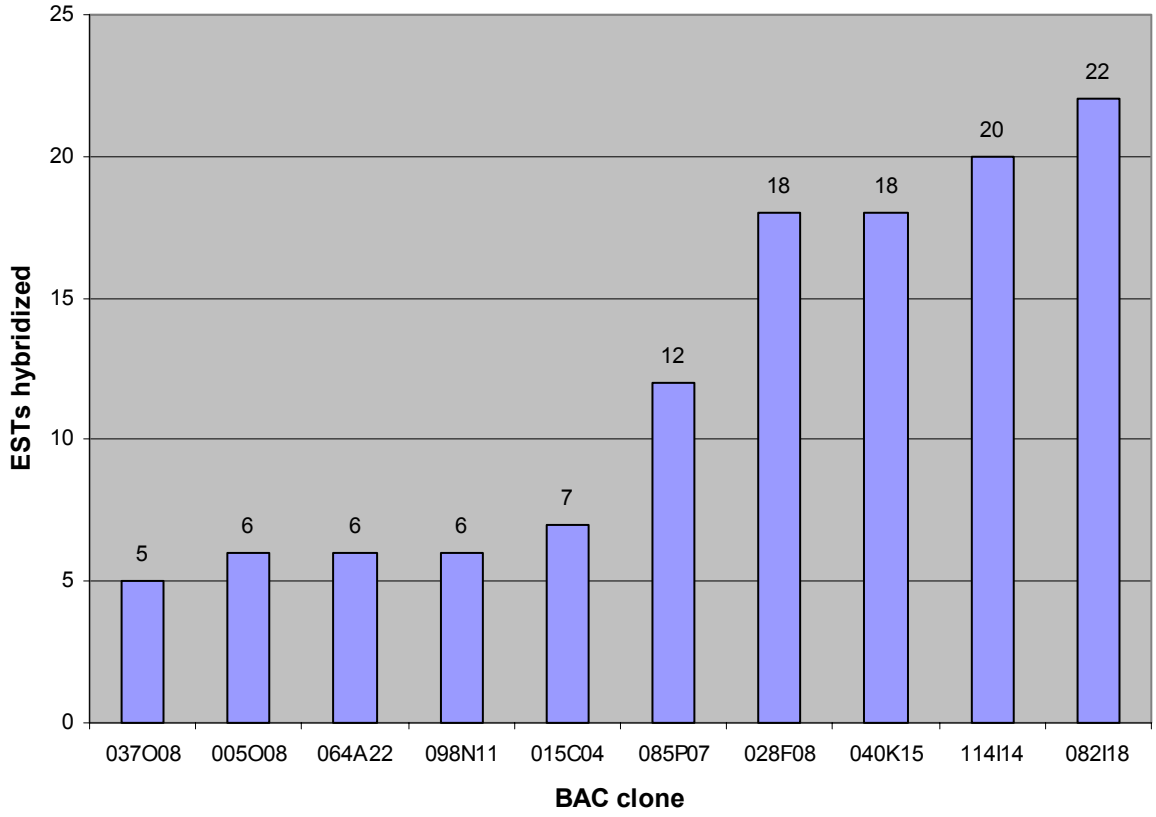

Figure 6. The ten BAC clones that hybridized to the most EST probes. Clones 082I18, 114I14, 028F08, and 085P07 are all anchored to the same two markers: AC55, which has two loci at G4 (AC55A, 1.8 cM) and G5 (AC55B, 14.6 cM), and LF11, located on linkage group 6 of the Myrobalan plum P.2175 X ('Garfi' almond X 'Nemared' peach) map, at 86 cM.

Chapter Three

Internal heat necrosis (IHN) of potato—a review

Abstract

Internal heat necrosis (IHN) is an internal physiological disorder of potatoes characterized by necrotic patches of parenchymal tissue inside the vascular ring. It has been described in the literature since the early 19th century, albeit under several different names. IHN has become a more severe problem since the late 20th century, since the introduction of the popular and high-yielding chipping cultivar 'Atlantic', which is highly susceptible to IHN. Several environmental factors play a role in the manifestation of the disorder. High day and night temperatures early in the growing season, combined with low rainfall, have been shown to increase the frequency and severity of the disorder, particularly in 'Atlantic'. Much research has been done on the ability of calcium to ameliorate IHN. Some of this work strongly suggests an important role for calcium in the development of IHN, but due to the varying conditions of the different experiments, it is difficult to interpret the overall results, and other soil nutrients are probably important as well. The biochemistry and genetics behind IHN have historically been the least-researched facets of the problem. Recent work has shown that enzymes involved in protecting cells from oxidative damage may be involved, and heritability studies have demonstrated that broad-sense heritability for IHN resistance is high.

Introduction

Necrosis of potato tubers has been reported in the scientific literature since the early 20th century (reviewed in Larson and Albert 1945). While S. Burr identified pathogenic bacteria in necrotic lesions of tubers in 1928, an in-depth study of non-pathogenic necrosis was not published until much later (Larson and Albert 1945). This report included a thorough, illustrated description of 'physiological internal necrosis', including histological studies at the cellular level. In addition, Larson and Albert reported on the effects of soil type and nutrition, soil temperatures and moisture, and different cultivars on the manifestation of internal necrosis.

Many different names have been given to non-pathogenic necrosis of potato tubers, including internal rust spot, internal browning, physiological internal necrosis, internal brown fleck, chocolate spot, and internal brown spot, or IBS (Sterrett et al. 1991a and references therein, Davies 1998). It is likely that these reports described the same or similar disorders, but variation due to cultivars and environments has been significant. In addition, there has been a relative lack of communication between different researchers (Sterrett and Henninger 1997).

Internal heat necrosis or IHN, as it will be referred to hereafter in this review, has received more serious attention since the introduction of the cultivar 'Atlantic' (Webb et al. 1979). 'Atlantic' has the desirable qualities of high yield potential, high specific gravity, wide adaptation, and disease resistance (Webb et al. 1979, Sterrett and Henninger 1997), and in North Carolina, 'Atlantic' is an important source of early-season chipping potatoes, as it comprises 70-75% of total commercial acreage (G.C. Yencho, personal communication). However, 'Atlantic' is also highly susceptible to IHN. Each year, as much as 11% of the

'Atlantic' acreage in North Carolina and Virginia is left unharvested due to IHN (Sterrett and Wilson 1990). Despite a reduction in acreage of 'Atlantic' due to IHN susceptibility (Henninger et al. 2000), its attractive qualities have resulted in its persistence. 'Atlantic' has set a standard for yield and dry matter content, particularly amongst round-white chipping potato cultivars, while its susceptibility to IHN has stimulated research into the causes for and solutions to the disorder, which also impacts a number of other important cultivars, including Yukon Gold, a popular yellow-fleshed table-stock variety, and 'Keuka Gold', a new yellow-fleshed cultivar from Cornell University.

Temperature and Moisture

It has long been known that the manifestation of IHN has a large environmental component. Larson and Albert (1945) reported more severe IHN in years with higher than normal temperatures, and lower than normal precipitation. Location also has a significant effect on IHN (Sterrett et al 1991a, 1991b, Henninger et al. 2000, Sterrett et al. 2003, Haynes et al., unpublished data), as does time to harvest (Sterrett et al. 1991a, 1991b, Lee et al. 1992).

Attempts to quantify the effects of temperature and moisture on IHN were not made until relatively recently. A significant contribution was made by Sterrett et al. (1991a), who developed a visual rating scale for IHN. This scale ranges from 9 (no visible necrosis) to 1 (nearly all perivascular tissue necrotic), and allows for more accurate quantification of IHN severity. Heat-sum models for the prediction of potato yield have been available for some time (reviewed in Sterrett et al. 1991b). The majority of these models have imposed a penalty on excessively high temperatures, as they reduce dry matter accumulation in tubers.

A heat-sum model predicting yield and the progression of IHN in 'Atlantic' was published by Lee et al. (1992). This model included variables reflecting time to harvest, maximum daily temperatures, and maximum nighttime temperatures. The Lee heat-sum model was found to have a higher predictive power than a model including only time to harvest and daily maximum temperatures (r^2=0.5 and 0.27, respectively). Though high minimum temperatures were shown to play a role in the progression of IHN, it was obvious that other environmental factors besides temperature were involved.

Sterrett et al. (1991a, 1991b) subsequently[1] developed more sophisticated environmental models for IHN incidence and severity.[2] In addition to time to harvest, the first models (1991a, listed hereafter as models 1 and 2) included a 'penalty' variable derived from the Lee heat-sum model (defined as the number of days until a period ≥ 3 consecutive days of negative heat units), as well as a variable reflecting the amount of rainfall in the first 60 days after planting (DAP). The predictive ability of models 1 and 2 was similar to the Lee heat-sum model (r^2= 0.439 for IHN incidence, 0.637 for IHN rating), but comparisons between models 1 and 2, and the Lee heat-sum model are somewhat difficult since Lee et al. (1992) modeled IHN severity as the interval in days between the first appearance of IHN and declaration of off-grade (a mean IHN rating of 7 or less), whereas Sterrett et al. (1991a) modeled severity as the average IHN rating of all tubers examined. In any case, models 1 and 2 demonstrated that cooler temperatures and higher rainfall early in the growing season could reduce the incidence and improve the mean severity rating of IHN.

[1] The heat-sum model of Lee et al. was developed before this research was published (see Sterrett et al. 1991a, but not published until 1992.

[2] A model for the distribution of necrosis within the tuber was also developed, but IHN distribution has not been widely used as a measure of the disorder.

The second group of models (1991b, hereafter listed as models 3 and 4) utilized a two-stage method of prediction. Model 3 predicted the days to first trace (first occurrence) of IHN, using as variables the slope of the regression line of the heat-sum model of Lee et al. (1992), the 'penalty' variable utilized in models 1 and 2, and the number of rain events in the first 60 DAP. This model showed high predictive ability ($r^2 = 0.855$). Model 4 predicted the number of days until a planting was considered offgrade due to IHN, and utilized the penalty variable, the number of rain events in the first 10 days after first trace, and the percentage of large tubers (> 64mm) at first trace. This model had even greater predictive power than model 3, with r^2=0.981. Taken together, models 3 and 4 demonstrated the importance of temperature during relatively early periods of tuber development. A warm spring, particularly with more than three consecutive days of hot weather (>25C maximum and 21C minimum temperatures), contributes greatly to the occurrence of IHN. Rainfall also plays an important role, but the number of rain events is important, not the total amount of precipitation. Sterrett et al. (1991b) suggested that rain events may be important for reducing light intensity and/or temperature, and/or increasing humidity rather than supplying soil moisture. The significance of large tubers in model 4 has been attributed to environmental conditions conducive to lower tuber set, and therefore larger tubers. All of the models developed by Sterrett et al. (1991a, 1991b) are useful both as predictive tools for determining harvest dates to minimize losses due to IHN, and as guidelines for experiments in controlled environments (e.g. Phytotron) to determine more precisely the role of the environment in the expression of IHN, as well as the interaction between genotype and environment.

Soil Fertility/The Calcium Connection

Variation in soil type and fertility is an obvious component of environmental variation, and research into IHN has often focused on these areas. Larson and Albert (1945) suggested that sandy soils are partly responsible, but no other research has been done specifically observing IHN in different soil types. Much research has been done, however, on the role of calcium in IHN. Greenhouse studies investigating the role of calcium in necrosis of potato tubers (Kelly and Christiansen 1970, Collier et al. 1978) found a link between low calcium levels and tuber necrosis. Tzeng et al. (1986) were the first to perform a field study of the effects of calcium applications on IHN, using 'Russet Burbank' as a test cultivar. Results showed that increasing the rate of calcium application did result in increased tuber peel calcium concentration, which was highly negatively correlated with the incidence of IHN ($r = -0.754$ over two seasons). A similar study by Sterrett and Henninger (1991), utilizing 'Atlantic' and three other cultivars, found that more susceptible cultivars generally had lower tuber calcium levels, with a correlation between tuber calcium (minus peel) and IHN similar to that of Tzeng et al. (1986) ($r = -0.785$). However, levels were significantly different between only two cultivars, 'Atlantic' and 'Superior'. In comparison to the results of Tzeng et al. (1986), increased calcium supplementation had no effect on tuber peel calcium (and an inconsistent effect on medullary calcium), though strangely, it did have an effect on IHN incidence. The similarities in results between these two studies are so consistent that the marked differences that do exist are likely due to genetic and environmental factors. Different potato genotypes have been shown to vary greatly in their ability to take up calcium (Bamberg et al. 1993). In addition, Simmons and Kelling (1987) have shown that potatoes grown in different soils vary in their response to increased calcium fertilization and these

experiments were conducted in very different environments (Wisconsin for Tzeng et al. (1986), and Virginia and New Jersey for Sterrett and Henninger [1991]).

What roles could calcium play in the manifestation of IHN, and how could calcium interact with other environmental factors shown to be involved? Calcium has important roles in three main areas (Hopkins 1999): cell division, including formation of the mitotic spindle and middle lamella; maintaining membrane integrity and function; as a secondary messenger involved in a number of metabolic processes, including fruit ripening (Llop-Tous et al. 2002, Leclercq et al. 2005), seed development (Anil et al. 2003), response to abiotic stresses such as heat, cold, drought, and high salt (Jiang and Huang 2001, Llop-Tous et al. 2002, Chehab et al. 2004, Abbasi et al. 2004), and attack by pathogens (reviewed by Lee and Rudd 2002).

Microscopy studies of necrotic tuber tissue by Larson and Albert (1945) showed disorganization of cells at the perimeter of necrotic areas, a possible indication of disruption of cell division in these regions. Monk et al. (1989) used electron spin resonance (ESR) to investigate necrotic tuber tissues and found that free radicals are produced upon necrotic tissue formation. They suggested that calcium deficiency in these tissues leads to a loss of membrane integrity and resultant free radical production and necrosis, but provided no direct evidence that this is the case.

Calcium deficiency can be linked to temperature and water stresses. Calcium movement in plants is through the xylem (Bangerth 1979), and as a result follows water potential gradients. Leaves have a lower water potential than tubers, which explains why leaves have higher calcium content than tubers (Wiersum 1966). Under periods of heat and drought, water potential in leaves drops further, resulting in even higher concentrations of calcium in

the leaves, at the expense of decreased calcium concentration in the tubers. This condition could then set the stage for IHN, and could be a link between calcium and environmental stresses in the manifestation of the disorder (Davies 1998). Application of antitranspirants in the form of wax emulsion or spreader-sticker surfactants to the foliage of 'Atlantic' potato plants been shown to increase leaf water potentials as compared to tubers, even under conditions of water stress (Win et al. 1991). This increase in leaf water potential also resulted in higher tuber calcium concentrations (and lower leaf calcium), and a corresponding decrease in IHN incidence and severity.

Calcium may not be the only nutrient that influences IHN, and it may not even be the most important. A recent study by Haynes et al. (personal communication) analyzed the effects of calcium and nine other nutrients (P, K, Mg, S, Na, Zn, Mn, Cu, and Fe) on IHN incidence and severity, specific gravity (SG) and yield. The experiment was run over six location years, using 'Atlantic' and 18 interspecific breeding lines. In addition to using the standard general linear model technique to analyze the data, Haynes et al. employed the more robust CART (classification and regression tree) technique, particularly to fit nutrient data to levels of IHN incidence for each location-year. While IHN-resistant clones on average did have higher calcium concentrations than IHN-susceptible clones, levels of Mn, P, and S each explained more of the observed variation than did calcium.

Clearly, manifestation of IHN is controlled by many factors, including temperature, moisture, and various soil nutrients, with interactions between many if not all of these variables. An additional and even more complex group of factors impacting the expression of IHN are the genes responsible for IHN resistance and/or susceptibility, and their interactions with each other and the other variables already discussed.

Genetics

The genetic component of IHN has been the least-studied element of the disorder. Multiple studies have observed considerable variation in IHN sensitivity between cultivars (Larson and Albert 1945, 1949, Ellison 1953, Wolcott and Ellis 1959, Sterrett and Henninger 1991). Most of these studies have found that potato cultivars fall into three broad classes: consistently resistant; consistently susceptible; variably susceptible/resistant. Consistently susceptible varieties vary in their severity. 'Atlantic', for example, is consistently a highly susceptible cultivar, with Henninger et al. (2000) reporting an IHN incidence of 53% averaged over two locations and three seasons. 'Mohawk', an older cultivar, had a 'mean necrotic index' (a 0-100 scale no longer used) of 9.6 over five seasons (Larson and Albert 1949). Cultivars that are variably resistant or susceptible are greatly affected by environmental conditions by year (Wannamaker and Collins 1992), location, or both (Henninger et al. 2000, Haynes et al., unpublished data). The well-known cultivar 'Kennebec' is one example of a clone that displays highly variable IHN symptoms (Wolcott and Ellis 1959).

This wide range of susceptibility to IHN, and its variable incidence in some genetic backgrounds, suggests that it is quantitatively inherited, with a large degree of genetic by environmental (G X E) interaction. As far as single genes are concerned, research has been done on the possible contribution to IHN of polyphenol oxidase, but no connection was found (G.C. Yencho, unpublished data). Davies and Talbot (1989) analyzed enzymatic activity of potatoes grown under low-calcium conditions, and showed that the IHN-resistant cultivar 'Desiree' had higher activities of superoxide dismutase, dehydroascorbate reductase,

and peroxidase than the IHN-susceptible clone 10337de40. These enzymes are involved in the elimination of free radicals, which are produced in higher numbers under stressful conditions.

Two recent studies have provided more information on the genetics of IHN. Henninger et al. (2000) utilized 4 cultivars and 15 breeding lines to quantify the broad-sense heritability (H) for IHN incidence and severity, SG, and yield, and to quantify any correlation between these three traits. Estimates of H were 0.83 for incidence and 0.85 for severity in size III (64-83mm diameter) tubers. Broad-sense heritability was also high for SG (of size III tubers) and total yield (0.92 and 0.86, respectively). These high estimates of H indicate that though there is a significant environmental component of IHN, as well as a great deal of G X E interaction, there is a significant amount of genetic control over the trait.

Earlier work by Sterrett et al. (1991a) revealed significant correlations between various measures of IHN (incidence, rating, and distribution) and yield, and Iritani et al. (1984) have observed increased IHN amongst tubers with high SG. These correlations suggested that perhaps high yields and SG, both hallmarks of 'Atlantic', come at the expense of susceptibility to IHN. However, IHN and yield are both more significantly correlated with time to harvest (Sterrett et al. 1991a), which suggests a confounding of yield and IHN. In addition, Henninger et al. (2000) found no correlation between either IHN and yield, or IHN and SG. These results suggest that it is possible to breed a low-IHN, high-SG, high-yield potato cultivar, one that could replace 'Atlantic'. Specific gravity is an important trait because it reflects the dry matter content of tubers. More dry matter equates to fewer tubers being needed to produce a given weight of potato chips, and less oil uptake. However, amongst the 19 clones analyzed by Henninger et al. (2000), all representing conventional *S.*

tuberosum germplasm, only two clones had higher mean SG than 'Atlantic'. These clones both had lower incidence of IHN than 'Atlantic', indicating they could be crossed with it to produce superior progeny, but Haynes et al. (unpublished data) have suggested that 'Atlantic' is not an ideal breeding parent; its progeny are much more likely to be susceptible to IHN. A potential solution is to expand the germplasm base through the use of wild potato species. From a population of 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids, Sterrett et al (2003) evaluated 88 clones for IHN incidence and severity, SG, and yield. 'Atlantic' was included as an IHN-susceptible check, and as a comparison standard for SG and yield. Results corroborated the findings of Henninger et al. (2000), in that IHN and SG were not correlated. In addition, clones were identified that had much lower incidence of IHN, and equal or greater SG than 'Atlantic'; these clones were also reliably IHN-resistant across environments, suggesting that exotic germplasm can help provide a stable genetic solution to the problem of IHN.

Summary and Conclusion

Research over the years has not been able to pinpoint the mechanisms behind IHN in potato, but it has been able to identify several of the contributing factors. Temperature, water relations, and soil nutrients have all been shown to have important effects, as well as the genetic complement of the potato itself. Interaction between these factors makes it difficult to quantify their importance, but now that many of the pieces of the puzzle have been identified, it is now possible to design new experiments that can disentangle these interactions. This separation of effects will allow each to be more accurately quantified, and

will help in identifying the physiological and genetic mechanisms behind internal heat necrosis.

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Chapter Four

Identification of amplified fragment length polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids

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Identification of amplified fragment length polymorphism (AFLP) markers associated with internal heat necrosis (IHN) in 4x-2x *Solanum tuberosum* x *S. phureja-S. stenotomum* hybrids.

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Abstract

Internal heat necrosis (IHN) is a physiological disorder of potato tubers resulting in the discoloration of parenchymal tissue. Environmental conditions and the large acreage of the IHN-susceptible cultivar 'Atlantic' result in significant losses due to the disorder in the mid-Atlantic states and Florida. A combination of bulked segregant analysis and AFLP marker technology was used to search for molecular markers for IHN in a population of tetraploid 4x-2x *S. tuberosum* x *S. phureja-S. stenotomum* (*tub* x *phu-stn*) hybrids. These clones are being used to breed an IHN-resistant, high specific-gravity replacement for 'Atlantic'. One marker associated with IHN resistance, potPCR13-HindIII-R, was identified in two small test populations where it explained 69.9% and 64 % of the observed variation for IHN incidence and severity, respectively. This marker showed strong sequence homology to calcium-dependent protein kinases (CDPKs), reinforcing prior evidence of the importance of calcium to the manifestation of IHN. When tested on a different set of clones from a combining ability study for IHN, this marker was no longer significant. However, a second putative marker from the BSA-AFLP screen (potPCR31-A) that was not statistically significant in the small populations, was significantly associated with IHN susceptibility in this second population, and the regression models of potPCR-31-A on IHN severity and incidence explained 17.8% and 18.6% of the phenotypic variation, respectively. This marker shows homology to plastid terminal oxidases (PTOXs), which are involved in desaturation of carotenoids, and may have a role in protecting biomolecules under oxidative stress. The small sample sizes used in marker development and initial testing may have resulted in these markers being significant predictors only in certain populations. It is also possible that, given the quantitative nature of IHN, only a subset of all IHN-associated loci may be necessary for

the disorder to be manifest. This research is the first molecular marker study of the genetics of IHN, and has set a foundation for future studies.

Introduction

Internal heat necrosis (IHN) is an internal physiological disorder of potato (*Solanum tuberosum* L.) tubers resulting in necrotic patches of parenchymal tissue inside the vascular ring (Larson and Albert 1945, Henninger et al., 1979). The necrotic patches initially appear at the apical end of the tuber, but in severe cases the affected area can encompass the entire parenchyma (internal to the vascular ring). Similar or identical necroses have been given various names by different researchers, including physiological internal necrosis (Larson and Albert 1945), internal brown spot (Tzeng et al. 1986, Davies 1998), internal browning, internal brown fleck, and chocolate spot (Sterrett and Henninger 1997 and references therein). It is likely that these reports are describing the same or similar disorders, but variation due to cultivars and environments has been significant, and, to date, the research community has not established a uniform terminology regarding this disorder (Sterrett and Henninger 1997).

'Atlantic', the most important chipping cultivar produced in the US because of its high yield, high specific gravity, and broad adaptation, is highly susceptible to IHN (Henninger et al. 1979). In the mid-Atlantic states (NJ, MD, VA, and NC) and Florida, whose production comprises a significant segment of the spring potato market utilized for chips, 'Atlantic' accounts for roughly 70% of the chip-stock potatoes produced. Because 'Atlantic' is the dominant variety produced and it is highly susceptible to IHN, this disorder is one of the most significant production constraints in the mid-Atlantic region. Sterrett and Henninger (1997) have studied IHN extensively. They developed an IHN severity rating system based on a scale of 1 to 9, with 1 being the most severe (Figure 1). Tubers with an IHN rating of 7 or lower are considered off-grade and loads with greater than five percent of

US No. 1 potatoes with an IHN rating of 7 can be rejected by chip processors (Sterrett et al. 2003, USDA-AMS 1991). It is not uncommon for entire loads of potatoes to be rejected by processors due to high levels of IHN, and it has been estimated that as much as 11% of the acreage of 'Atlantic' is not harvested each year in North Carolina and Virginia due to occurrence of this disorder (Sterrett and Wilson 1990). The average annual value of the potato crop in the mid-Atlantic states and Florida during the years 2001-2003 was over $174 million (USDA-NASS 2004), thus losses due to IHN are of great significance.

Little is known about the causes of IHN, and this has limited research into its control. The incidence and severity of IHN are highly dependent upon environmental conditions. Sterrett et al. (1991) developed a two-stage mathematical model to predict the occurrence of IHN in 'Atlantic' in the mid-Atlantic region. The major components of the model are heat accumulation (day and night temperatures) in the early part of the growing season (before 60 days after planting), and the number of rainfall events throughout the season. Incidence and severity of IHN can be reduced by later planting dates and/or frequent rainfall events. However, planting later in the season reduces yields, and since irrigation is not widely practiced in the mid-Atlantic states (G. C. Yencho, personal communication), farmers do not generally plan on more frequent precipitation. A review of possible methods of control by Sterrett and Henninger (1997) concluded that none were truly satisfactory, and that the best method of dealing with IHN would be to breed a replacement for 'Atlantic'.

Henninger et al. (2000) demonstrated that susceptibility to IHN is, to a large extent, under genetic control. Using a set of 4x-2x *S. tuberosum* x *S. phureja-S. stenotomum* (*tub* x *phu-stn*) hybrids Henninger et al. (2000) obtained broad-sense heritability estimates of 0.83 (95% CI .68, .93) and 0.9 (95% CI .78, .96) for IHN incidence and severity, respectively. A

population of tetraploid clones derived from the *tub* x *phu-stn* hybrids (hereafter the IHN progenies), which contains stable resistance or susceptibility to IHN, is currently being used to develop a high-specific gravity, IHN-resistant replacement for 'Atlantic' (Sterrett et al. 2003).

To supplement the traditional breeding approach, it is desirable to develop DNA-based markers to select for IHN resistance. DNA markers can be used to improve the efficiency and accuracy of selection in traditional breeding programs. For example, Willcox et al. (2002) demonstrated that marker-assisted backcrossing was as effective as traditional backcrossing, when breeding for southwestern corn borer resistance in maize, a quantitative trait. In this case, marker-assisted selection (MAS) was more expensive than conventional selection (CS) due to the costs of initial marker development, but the authors noted that with known markers on hand, costs for MAS would have been much lower than for CS, making future use of the markers very attractive. In wheat, MAS has been used to successfully incorporate the *Sf-2* gene for resistance to stem rust, a trait that is recessively inherited, with an environmentally variable phenotype that is time-consuming to document in the field (Hayden et al. 2004). Because IHN exhibits no symptoms other than within the tuber (Larson and Albert 1945, Tzeng et al. 1986), traditional field-based screening for the disorder requires a full growing season, as well as considerable time and space. This is compounded by the fact that the manifestation of IHN is highly dependent upon environmental conditions (Wannamaker and Collins 1992, Lee et al. 1992). Several years of data from multiple locations are necessary to obtain an accurate and reliable estimate of a clone's resistance or susceptibility to IHN. DNA marker analysis would only require a small amount of leaf tissue from young plants and a few pieces of common laboratory equipment, resulting in a

considerable cost savings. A further advantage of MAS is that, if the markers themselves appear to be expressed sequences, they can be considered candidate genes and serve as the basis for more in-depth study of the genetics and biochemistry behind the disorder.

When developing molecular markers, it is desirable to rapidly and cost-efficiently screen large areas of the genome for polymorphism. Amplified fragment length polymorphism (AFLP) technology (Vos et al. 1995), a PCR-based method that has the ability to reproducibly generate large numbers of DNA fragments without prior sequence knowledge and at minimal cost, is an attractive method for marker development. AFLP technology can be combined with a technique termed bulked segregant analysis (BSA) to increase the likelihood of detecting polymorphisms linked to the trait of interest (Michelmore et al. 1991). By creating pools or 'bulks' of DNA from individuals with a common trait, the DNA samples are enriched for the sequences of interest. Our objective in this study was to use AFLP markers and bulked segregant analysis techniques to identify markers associated with IHN resistance and/or susceptibility. We hope to use these markers for breeding purposes, and to identify potential candidate genes for future studies focused on understanding the physiological basis of IHN.

Materials and Methods

Field evaluation of IHN populations

A multi-state study to determining the genetic basis of resistance to IHN in clones of the IHN progenies was initiated in 1999 at three locations: Plymouth, NC, Painter, VA, and Bridgeton, NJ (Sterrett et al. 2003). The experimental designs used to evaluate yield, specific gravity, and resistance/susceptibility to IHN in each of these locations were described by

Henninger et al. (2000) and Sterrett et al. (2003). IHN rating protocols were as follows. Twenty size class III tubers (64 - 83 mm) of each clone were quartered longitudinally, and rated for IHN incidence and severity according to the IHN rating scale developed by Sterrett and Henninger (1997) (Fig. 1). In plots where 20 size class III tubers were not available, the largest class II tubers (48 – 64 m) were examined to obtain 20 tubers per clone.

AFLP generation and conversion

In the fall of 2002, DNA was extracted from the first 17 clones of the IHN progenies using a CTAB-based protocol from Michigan State University (B. Sosinski, personal communication). The five most IHN-susceptible and the five most IHN-resistant clones of this group (determined by North Carolina field data, Figure 2) were selected for bulked segregant analysis. This group of ten individuals was designated IHN Population 1. BSA was performed using Vos et al.'s (1995) AFLP technique modified for use with fluorescently labeled EcoRI primers and a LI-COR 4000L single-dye automated sequencer (Lincoln, NE). Preamplification reactions were performed on each member of IHN Population 1, and aliquots of these reactions were pooled to generate the IHN-resistant and IHN-susceptible bulks. The bulks were then screened with 192 selective primer combinations. Putative polymorphisms were verified by rerunning reactions on the individuals of the bulks.

In order to clone the AFLP bands of interest and convert them to sequence-specific markers, verified primer combinations were run a third time (using non-pooled DNA from one IHN-resistant and one IHN susceptible clone) using ^{32}P-labeled primers, and resolved on an 8% polyacrylamide gel. The gel was sandwiched between Whatman 3MM blotting paper (Florham Park, NJ) and plastic food wrap (Reynolds Metals Company, Richmond, VA), and

dried. Following overnight exposure at -70° C on X-ray film, 37 polymorphic fragments were excised from the gel and eluted overnight in 50 uL of water. Five uL of this elution was used as template for PCR reamplification of the fragments, which were then ligated into pGEM-T Easy (Promega, Madison, WI), and used to transform *E. coli* strain DH5-∀ (Invitrogen, Carlsbad, CA) via electroporation.

To obtain reliable sequence data, at least four clones were sequenced for each of the successfully reamplified fragments. Sequencing was performed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) using T7 primers (5'AATACGACTCACTATAG-3') from Operon Biotechnologies (Huntsville, AL). Due to the presence of co-migrating bands, 60 unique sequences were identified from the 34 sequenced fragments. All unique sequences were used to query the GenBank database via the BLAST (Basic Local Alignment Search Tool) algorithm. A total of 64 PCR primer pairs were designed for these unique sequences, either by hand or by the use of the Primer3 module of the Genomax software suite (Informax/Invitrogen, Carlsbad, CA).

Marker development

The new primer pairs were tested for amplification and polymorphism using the same clones demonstrating polymorphism in the radioactive AFLP analyses. All but three of the successful primer pairs appeared to be monomorphic when amplification products were separated via electrophoresis through a 2% agarose gel. Therefore, resequencing was performed on the remaining fragment, i.e. if the fragment from the susceptible individual was sequenced first, then the fragment from the resistant individual was sequenced, and vice versa. This resequencing detected a number of point mutations between the resistant and

susceptible individuals, which resulted in the identification of two additional markers; these could be detected by restriction digestion following PCR. Markers were selected for further testing if at least three of the plants in one bulk had a genotype different from the other bulk. Once a set of putative markers had been obtained, we tested them on an additional six IHN-resistant and three (two from the IHN progenies plus 'Atlantic') IHN-susceptible clones, which we labeled IHN Population 2 (Figure 3). Table 1 lists and describes the markers that met the criterion described above in both of these subpopulations.

Marker testing

In 2004, a large-scale general combining ability study was performed using three members of the original IHN progenies as parents, along with 'Atlantic' and four advanced selections from the USDA breeding program in Beltsville, Maryland. The study consisted of 16 families (4 males, 4 females in a NC Design II crossing design), each with 20 clones per family. The five putative IHN markers described in Table 1 were evaluated in a more rigorous experiment using all 20 clones of a family (B2460) exhibiting a high frequency of IHN, plus two individuals (one IHN resistant and one IHN susceptible clone when possible) from each of the remaining families (except one each from families B2459 and B2461), for a total of 48 clones (Figures 4 and 5). Statistical analyses of the relationships between marker genotypes and IHN severity or incidence were performed using the PROC CORR, PROC GLM, and PROC REG commands of SAS version 8 (SAS, Cary, NC). IHN Populations 1 and 2 were combined for these analyses. Multiple regression of all markers on IHN severity was analyzed first. Using a form of manual stepwise regression, highly insignificant markers (P-value > 0.25) were dropped from the model, until only markers with P-values > 0.05

remained (Tables 2A, B). Means and least significant differences for each of the significant marker classes were generated using the 'MEANS' and 'lsd' (Fisher's lsd test) options of PROC GLM.

Results

The majority of the individuals comprising the population from which IHN (sub)Populations 1 and 2 were selected were IHN-resistant (data not shown). This fact, combined with the small initial population size, limited the size of our bulks to five individuals each. As can be seen from Figures 4 and 5, most clones from the combining ability study also displayed relatively little IHN. Even in family B2460, where both parents were IHN-susceptible (data not shown), 60% of the clones had an IHN incidence of less than 20%. Distributions such as these are evidence of a quantitative trait, but one that is perhaps controlled by only a few genes.

For IHN Populations 1 and 2, marker potPCR13-HindIII-R (Table 2A, Figure 6) was the only significant predictor of IHN severity or incidence ($p < 0.0001$ for either variable). This marker was associated with an average decrease in IHN incidence of more than 52 percent, and/or an average increase in IHN rating of 1.88. Since IHN severity and incidence are highly correlated ($r = -0.988$), it is not surprising that this marker had an effect on both characteristics of the disorder. The sequence of potPCR13-HindIII-R bears significant homology to a calcium-dependent protein kinase (CDPK) from *Arabidopsis thaliana* (expect value = $1e^{-13}$, GenBank accession number BT003367).

The only significant marker identified for the IHN combining ability progenies was potPCR_31-A. It was associated with an average increase of 0.792 units of IHN severity (coefficient is negative because smaller scores denote more necrosis), and/or an average

increase of 27.9% in IHN incidence. Interestingly, this allele is a much larger PCR product (568 base pairs) than what was predicted (177 base pairs) on the basis of the primer design (Figure 7), but its strong amplification suggests it is not an artifact. The sequenced fragment exhibited high homology to a plastid terminal oxidase (PTOX) from tomato (expect value = $1e^{-90}$, GenBank accession number AF177979).

Amongst clones of IHN Populations 1 and 2, potPCR13-HindIII-R explained 69.9% and 64 % of the observed variation for IHN incidence and severity, respectively (Table 2A). Values of this magnitude are typical of 'major' QTL –quantitative trait loci— (Etienne et al. 2002). The regression models of potPCR-31-A on IHN severity and incidence (Table 2B) explain 17.8% and 18.6% of the phenotypic variation, respectively, amongst the 48 clones of the combining ability progenies. If we assume that IHN resistance/susceptibility is quantitative, then these estimates are similar to those obtained for 'minor' QTL for several other species, including cold sweetening in diploid potato (Menendez et al. 2002) sucrose content, yield, and quality in sugar beet (Schneider et al. 2002), and drought tolerance in rice (Lanceras et al. 2004).

Discussion

Potato is the most important vegetable crop produced in the U.S, and it is an important component of the eastern US crop production system. In Florida and the mid-Atlantic states, which supply much of the spring and summer crop potatoes in the US, the farm gate value of potato production was estimated to be over $174 million in 2003 (USDA-NASS 2004). Assuming that 5% of the crop is not harvested due to IHN damage, a conservative estimate according to Sterrett and Wilson (1990), losses due to IHN are valued at roughly $8.7 million

per year for the region. The development of IHN-resistant cultivars through cost-efficient marker-assisted selection presents an attractive method to combat these considerable losses.

From a visual assessment (observation of band patterns in agarose gels), markers potPCR5-EcoRI, potPCR13-HindIII, potPCR18 potPCR27, and potPCR31 appeared to be useful predictors of IHN incidence/severity in IHN Populations 1 and 2. When used as single variables in the model, all but potPCR31 were significant, and the class means for these markers (except potPCR31) were also significantly different (data not shown). However, when these markers were combined with potPCR13-HindIII-R in a multiple regression model, they were no longer significant, suggesting that they were correlated with potPCR13-HindIII-R. A test of this hypothesis revealed that the correlation between potPCR13-HindIII-R and potPCR27 was high ($r = -0.65$, p-value = 0.0025), and that the correlation between potPCR13_HindIII_R and potPCR5_EcoRI (both alleles) was not significant at $p < 0.01$, but was still substantial (p-values = 0.027 and 0.065, respectively). In effect, potPCR13-HindIII-R conveys the same information as the markers it is correlated with, and does so more effectively. Indeed, it was an effective marker amongst the IHN Population 1 and 2 clones tested, as it accounted for 69.9% and 64% of the variation for IHN incidence and severity, respectively.

When tested on the combining ability progenies, marker potPCR13-HindIII-R was not informative even though it showed strong association with IHN resistance/susceptibility with the first two sets of progenies. The failure of potPCR13-HindIII-R in the combining ability progenies may be due to one or both of two possibilities. First, the marker may represent a false positive, or an example of sampling error due to the relatively small population size used to establish our initial IHN-resistant and –susceptible DNA bulks. Vos

et al. (1991) used 14 to 20 individuals per bulk, and Bradeen and Simon (1998) used as many as 64 individuals, whereas we were only able to use five plants due to the small population size available initially. A second explanation may stem from the fact that IHN resistance/susceptibility is believed to be quantitative (G.C. Yencho, personal communication, Sterrett et al. 2003). It is possible that only a subset of all the loci controlling IHN are required for its manifestation, i.e. these markers may influence IHN only in certain populations. For example, 'Atlantic', though it is IHN-susceptible, carries the 'resistant' allele of potPCR13 (Figure 6), and was a parent in 4 of the 16 combining ability families, including B2460, the most IHN-susceptible family in which we conducted our marker tests. As a result, a large number of the progenies also carry the resistant allele, even though they may indeed be IHN-susceptible. The failure of markers potPCR5-EcoRI, potPCR18, and potPCR27 is probably due to the fact that they are correlated with potPCR13-HindIII-R. These results reinforce the fact that genetic markers are often only useful within the population that they are identified; in a different population, these same markers may simply not be segregating.

While this research has not been completely successful in obtaining AFLP markers linked to IHN resistance for use in a breeding program (i.e. the five markers were not informative enough for an 'at-a-glance' assessment of IHN susceptibility/resistance), we have identified two markers, potPCR13-HindIII-R and potPCR-31-A, that appear to be associated with IHN severity/incidence, and which bear strong similarity to functional genes that may provide further insights into the physiological and/or biochemical basis of IHN.

The sequence of potPCR13-HindIII-R bears significant homology to a calcium-dependent protein kinase (CDPK) from *Arabidopsis thaliana* (expect value = $1e^{-13}$, GenBank

accession number BT003367). Calcium-dependent protein kinases (CDPKs) play an important role in Ca-mediated signaling (reviewed by Ludwig et al. 2004). These proteins are involved in signaling during a number of metabolic processes, including fruit ripening (Llop-Tous et al. 2002, Leclerq et al. 2005) and seed development (Anil et al. 2003), response to abiotic stresses such as cold, drought, and high salt (Llop-Tous et al. 2002, Chehab et al. 2004, Abbasi et al. 2004), and attack by pathogens (reviewed by Lee and Rudd 2002). In potato, CDPKs have been shown to be involved in early stages of tuber development (Raíces et al. 2001, 2003). Much research has been undertaken to determine if calcium plays a role in IHN. A lower calcium content in tuber peel has been associated with an increase in IHN (Collier et al. 1980, Tzeng et al. 1986), and Davies (1998) showed a weak but significant correlation ($r = -0.34$) between total tuber calcium and incidence of IHN. In addition, Davies and Talbot (1989) showed that an IHN-susceptible selection (90% incidence under low calcium conditions) had less stable membranes and lower antioxidant activity than the resistant cultivar 'Desiree' (10% incidence under low calcium), both factors that could lead to tissue damage under conditions of low calcium supply. While a great deal of work remains to be done, CDPKs, and in particular the one encompassing potPCR13-HindIII-R, could provide the link between calcium and IHN, at least in certain genetic backgrounds.

Similarly, potPCR-31-A shows high homology to a plastid terminal oxidase (PTOX) from tomato (expect value = $1e^{-90}$, GenBank accession number AF177979). The basic function of PTOX appears to be oxidation of plastoquinone within the thylakoid membrane, resulting in consumption of molecular oxygen (Kuntz 2004). PTOX plays an important role in the desaturation of phytoene, a key step in the biosynthesis of carotenoids. Mutation of PTOX in *Arabidopsis* results in the variegated *IMMUTANS* phenotype, where developing

chloroplasts become photobleached (Aluru and Rodermel 2004). In tomato, PTOX has been shown to be impaired in the *ghost* mutant (Josse et al. 2000), whose fruits are pale due to a lack of lycopene accumulation in chromoplasts. The internal tissue of potato tubers is obviously devoid of either of these plastids, but it is possible that the protein encoded by potPCR31-A could function in amyloplasts to prevent oxidation of membrane components during periods of oxidative stress caused by high soil temperatures and/or water deficiency.

This research has provided a foundation for additional work to understand the genetics of IHN resistance/susceptibility. We will further test the five original markers on all the individuals of the IHN combining ability study, as well as develop new QTL mapping populations that will be developed from members of this study and/or members of the original IHN population. These tests will provide greater power to detect statistically significant associations between markers and IHN incidence/severity. Several of the point mutations identified after resequencing remain to be exploited, and may yet prove informative. In addition, we will repeat the BSA-AFLP screening using new bulks comprised of more individuals, to increase the robustness of our marker detection.

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Tables and Figures

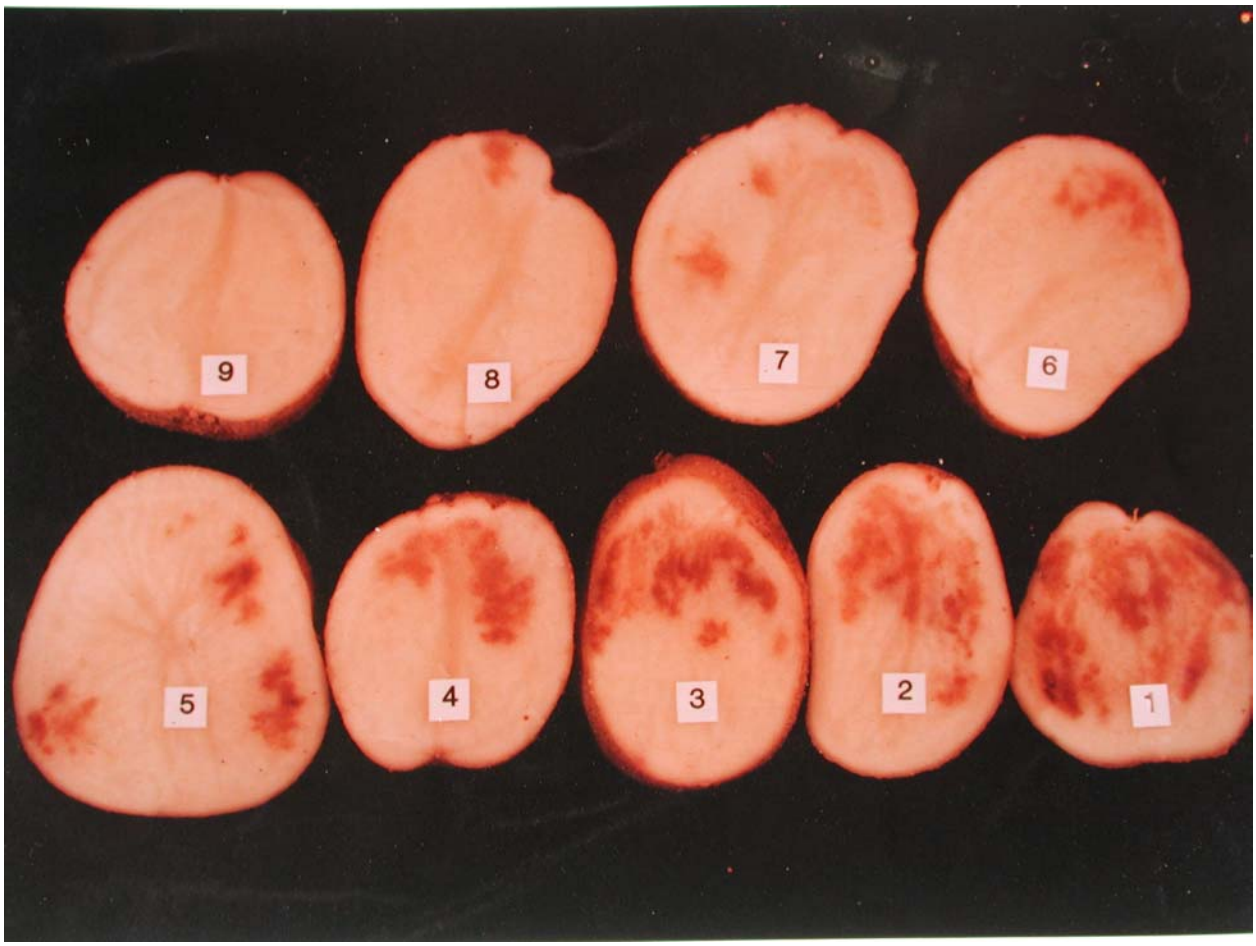

Figure 1. Internal heat necrosis in potato. Tubers are arranged to show selective grading system developed by Sterrett and Henninger (1997). A rating of 7 or lower is considered off-grade.

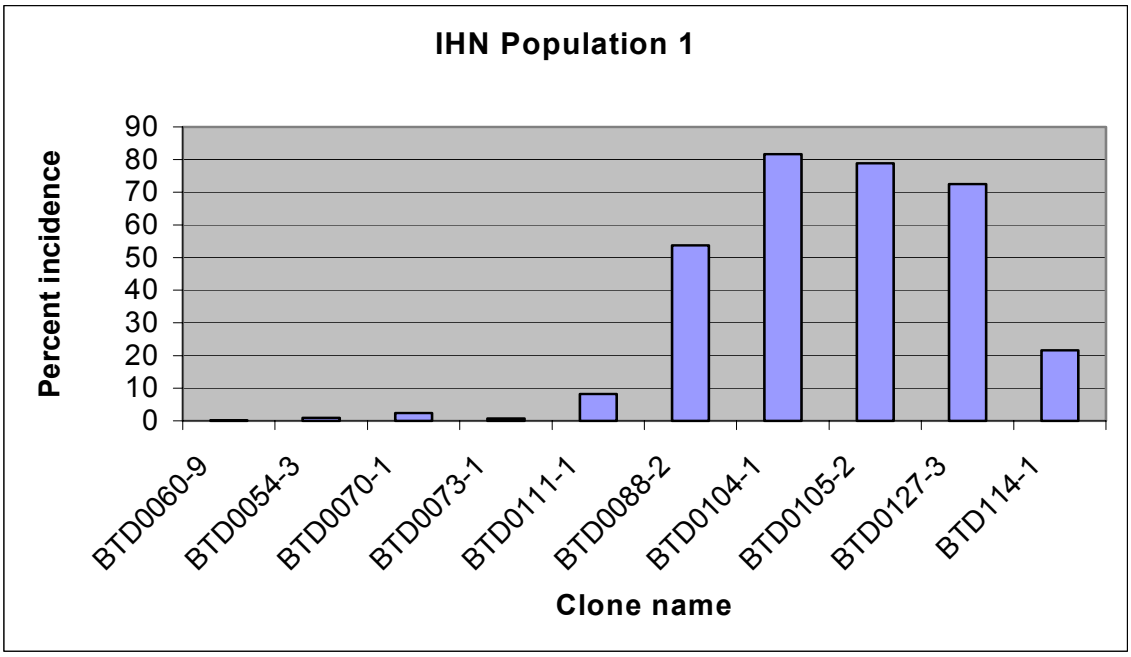

Figure 2. Ratings of IHN incidence of members of IHN Population 1, the population used for BSA. BTD0060-9 through BTD0111-1 were considered resistant, while BTD088-2 through BTD114-1 were considered susceptible.

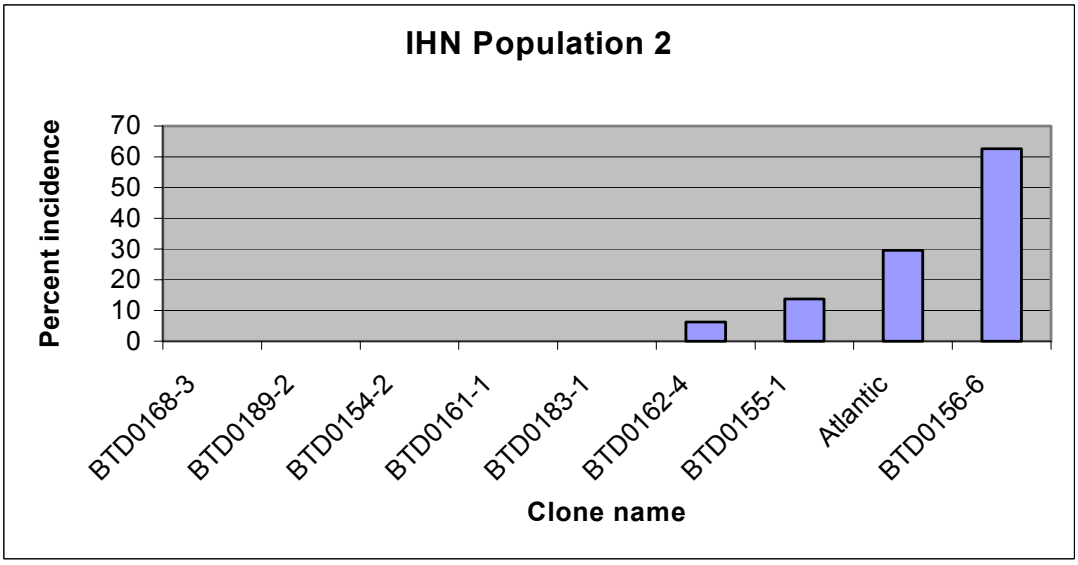

Figure 3. Ratings of IHN incidence of members of IHN 2, the population used for preliminary marker testing.

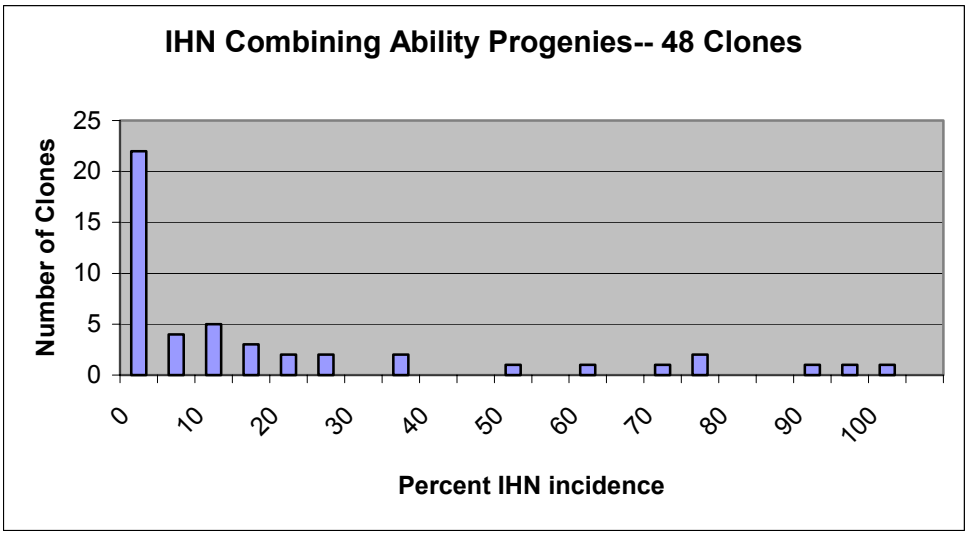

Figure 4. Histogram of the range of IHN incidence in the IHN combining ability progenies. Data is from North Carolina tests.

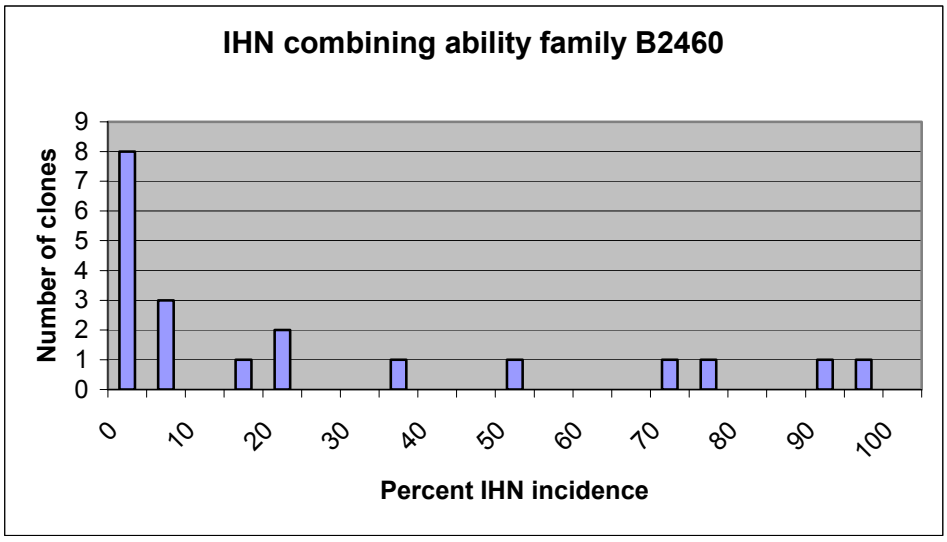

Figure 5. Histogram of the range of IHN incidence for combining ability family B2460, the one with the most IHN incidence.

Table 1. Putative markers identified for IHN resistance/susceptibility using the BSA-AFLP technique. Markers were considered associated with IHN if at least 3 members of each group (resistant/susceptible) had the same marker genotype, one differing from members of the other group.

Marker name	**Length (bp)**	**Homology (Genbank)**	**Description**	**Expect value**	**Notes**
PotPCR5_EcoRI	592	POL2_MOUSE	Retroelement	$3e^{-17}$	Cut with EcoRI
PotPCR13_HindIII	309	BT003367	*Arabidopsis thaliana* Ca-dependent protein kinase	$1e^{-13}$	Cut with HindIII
PotPCR18	285	BQ516321	*Phytophthora infestans*-challenged potato EST	$5e^{-63}$	
PotPCR27	332	CK285012	*Nicotiana benthamiana* EST	$9e^{-07}$	Two alleles (B,C)
PotPCR31	177				Three alleles (A,B,C)

Table 2. Final regression models for the markers tested on members of the combined IHN populations 1 and 2 (A), and for the IHN combining ability study (B). Correlation between IHN incidence and severity was extremely high: r = -0.957.

A.

Trait	**Model**	**P-value of regression coefficient**	**R^2 (adjusted)**
IHN Incidence	Mean(incidence)= 0.55 – 0.52(potPCR13-HindIII-R)	<0.0001	0.699
IHN Severity	Mean(severity) = 7.06 + 1.88(potPCR13-HindIII-R)	<0.0001	0.64

B.

Trait	**Model**	**P-value of regression coefficient**	**R^2 (adjusted)**
IHN Incidence	Mean(incidence)=.10315 + 0.279(potPCR31-A)	0.0013	0.1855
IHN Severity	Mean(severity) = 8.80235 – 0.792(potPCR31-A)	0.0018	0.1752

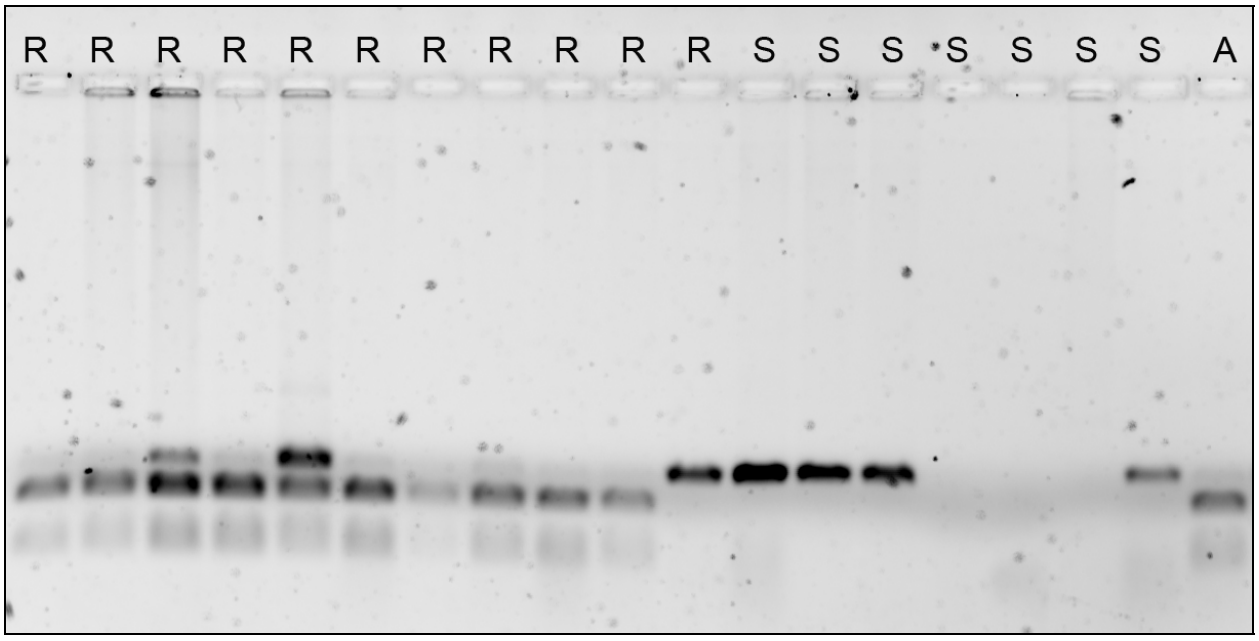

Figure 6. The marker potPCR13-HindIII-R, tested on members of IHN Populations 1 and 2. PCR products were digested with HindIII and separated via electrophoresis through a 2% agarose gel. A= 'Atlantic', R= resistant, S= susceptible. Polymorphism is not evident until after digestion with HindIII.

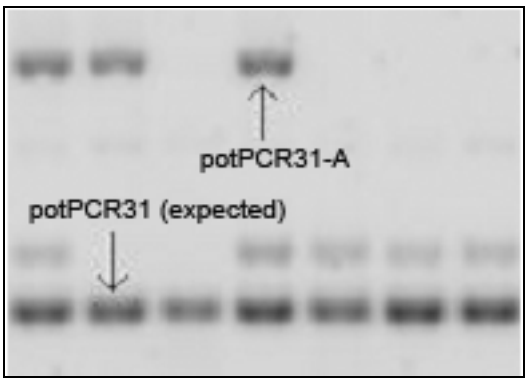

Figure 7. Marker potPCR31. Fragment A was polymorphic and informative amongst clones of the combining ability study.

Appendix

Peach ESTs genetically anchored to the physical map. 'No match' indicates no match with an expect value less than $1xe^{-9}$ was found in the GenBank nr database.

EST Name	Linkage Group	Location (cM)[1]	Trait association	Homology
PP_LEa0012O16f	1	0		NP_190365: *Arabidopsis* calcineurin-like phosphoesterase family
PP_LEa0012K02f	1	5.3		AAK26033: *Arabidopsis* unknown protein
PP_LEa0010I06f	1	16.2		NP_193325: *Arabidopsis* phosphatases pleiotropic regulator 1 (PRL1)
PP_LEa0009A14f	1	16.2		NP_187344: *Arabidopsis* transducin family protein
PP_LEa0013P13f	1	20.3		No match
PP_LEa0012C18f	1	23		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0012C18f	1	34.5	Sharka resistance[2], peach flesh color	NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0010I06f	1	35.4	Sharka resistance	NP_193325: *Arabidopsis* phosphatases pleiotropic regulator 1 (PRL1)
PP_LEa0009A14f	1	35.4	Sharka resistance	NP_187344: *Arabidopsis* transducin family protein
PP_LEa0013A14f	1	40.5	Sharka resistance, Evergrowing	AAK25976: *Arabidopsis* unknown protein
PP_LEa0009B20f	1	43	Evergrowing	AAB99745: *Triticum aestivum* hsp70 protein
PP_LEa0011C13f	1	43	Evergrowing	NP_196457: *Arabidopsis* expressed protein
PP_LEa0010P12f	1	54.3		No match
PP_LEa0011L02f	1	54.3		No match
PP_LEa0013F16f	1	54.3		NP_199101: *Arabidopsis* lipin family protein
PP_LEa0010K20f	1	54.3		NP_172261: *Oryza sativa* ribosomal protein L29 family protein
PP_LEa0013C10f	1	65		NP_194849: *Arabidopsis* DNA topoisomerase family protein
PP_LEa0009A08f	1	65.7		NP_187344: *Arabidopsis* transducin family protein
PP_LEa0013I08f	1	66.6		AAL26911: *Prunus persica* unknown protein
PP_LEa0013A14f	1	83.1	Petal color	AAK25976: *Arabidopsis* unknown protein
PP_LEa0012I23f	2	8.7		AAK16169: *Oryza sativa* unknown protein
PP_LEa0012C18f	2	17		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0011F23f	2	24	Almond shell hardness	NP_176511: *Arabidopsis* C2 domain-containing protein
PP_LEa0016M01f	2	24	Almond shell hardness	T50649: *Oryza sativa* elicitor-responsive gene 3

EST Name	Linkage Group	Location (cM)	Trait association	Homology
PP_LEa0016K20f	2	26	Almond shell hardness	NP_486113: *Nostoc sp.* glutamate N-acetyltransferase
PP_LEa0011I14f	2	38.5		NP_568159: *Arabidopsis* guanylyl cyclase-related (GC1)
PP_LEa0010O03f	2	38.5		No match
PP_LEa0008M11f	2	38.5		NP_196082: *Arabidopsis* aminotransferase class I and II family protein
PP_LEa0008M06f	2	41.6		No match
PP_LEa0011K19f	2	45.8		AAL67085: *Arabidopsis* unknown protein
PP_LEa0011F23f	2	49.3		NP_176511: *Arabidopsis* C2 domain-containing protein
PP_LEa0011P19f	2	49.3		NP_566056: *Arabidopsis* chloroplast signal recognition particle receptor protein
PP_LEa0016K20f	2	49.3		NP_486113: *Nostoc sp.* glutamate N-acetyltransferase
PP_LEa0016M01f	2	49.3		T50649: *Oryza sativa* elicitor-responsive gene 3
PP_LEa0011I14f	2	52.3		NP_568159: *Arabidopsis* guanylyl cyclase-related (GC1)
PP_LEa0010O03f	2	52.3		No match
PP_LEa0008M11f	2	52.3		NP_196082: *Arabidopsis* aminotransferase class I and II family protein
PP_LEa0008M06f	2	54.5		No match
PP_LEa0009H18f	3	36.4	Polycarpel, flower color	NP_563916: *Arabidopsis* vacuolar ATP synthase subunit C
PP_LEa0012C18f	3	31		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0012C18f	3	33.5	Polycarpel	NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0012O16f	3	33-63		NP_190365: *Arabidopsis* calcineurin-like phosphoesterase family
PP_LEa0009L20f	3	36.4	Polycarpel, flower color	NP_173299: *Arabidopsis* 3-phosphoserine phosphatase
PP_LEa0012E22f	3	36.4	Polycarpel, flower color	No match
PP_LEa0012N06f	3	36.4	Polycarpel, flower color	No match
PP_LEa0013A04f	3	41	Flower color	NP_193761: *Arabidopsis* TFIID 28kD subunit family protein
PP_LEa0013A21f	3	42.3	Flower color	No match
PP_LEa0012K19f	3	42.4	Flower color	NP_564018: *Arabidopsis* oligouridylate-binding protein, putative
PP_LEa0009B20f	3	117.2		AAB99745: *Triticum aestivum* hsp70 protein
PP_LEa0016G11f	4	1.8		No match
PP_LEa0016A20f	4	1.8		No match
PP_LEa0013M13f	4	1.8		No match

EST Name	Linkage Group	Location (cM)	Trait association	Homology
PP_LEa0013H23f	4	1.8		No match
PP_LEa0012O15f	4	1.8		No match
PP_LEa0012O21f	4	1.8		NP_177616: *Arabidopsis* nodulin family protein
PP_LEa0011F12f	4	1.8		No match
PP_LEa0012B20f	4	1.8		NP_567479: *Arabidopsis* expressed protein
PP_LEa0010M17f	4	1.8		No match
PP_LEa0010K11f	4	1.8		No match
PP_LEa0010I18f	4	1.8		No match
PP_LEa0010C17f	4	1.8		No match
PP_LEa0009N08f	4	1.8		NP_195026: *Arabidopsis* calcineurin B-like protein 10 (CBL10)
PP_LEa0009L10f	4	1.8		No match
PP_LEa0010B17f	4	1.8		No match
PP_LEa0010C09f	4	1.8		NP_565558: *Arabidopsis* expressed protein
PP_LEa0009H20f	4	1.8		No match
PP_LEa0009G20f	4	1.8		NP_194405: *Arabidopsis* hydroxyproline-rich glycoprotein family protein
PP_LEa0009I06f	4	1.8		AAB88874: *Prunus armeniaca* enoyl-CoA hydratase
PP_LEa0008N06f	4	1.8		No match
PP_LEa0008L17f	4	1.8		No match
PP_LEa0009A05f	4	1.8		No match
PP_LEa0012A23f	4	3		No match
PP_LEa0012A24f	4	3		NP_566732: *Arabidopsis* dienelactone hydrolase family protein
PP_LEa0012K18f	4	3		BAB63467: *Oryza sativa* putative aspartate aminotransferase
PP_LEa0009P04f	4	5.4		AAL09741: *Arabidopsis* unknown protein
PP_LEa0009L15f	4	5.4		No match
PP_LEa0009H20f	4	5.4		No match
PP_LEa0009P04f	4	42.3		AAL09741: *Arabidopsis* unknown protein
PP_LEa0009H20f	4	42.3		No match
PP_LEa0012C18f	4	43		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0012C18f	4	48		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0013B18f	4	53.1	Flesh adherence to stone	AAD13037: *Phaseolus vulgaris* NBS-LRR-like protein cD8
PP_LEa0013B18f	4	73.5		AAD13037: *Phaseolus vulgaris* NBS-LRR-like protein cD8
PP_LEa0012C18f	5	8		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0016G11f	5	14.6		No match
PP_LEa0016A20f	5	14.6		No match

EST Name	Linkage Group	Location (cM)	Trait association	Homology
PP_LEa0013M13f	5	14.6		No match
PP_LEa0013H23f	5	14.6		No match
PP_LEa0012O15f	5	14.6		No match
PP_LEa0012O21f	5	14.6		NP_177616: *Arabidopsis* nodulin family protein
PP_LEa0011F12f	5	14.6		No match
PP_LEa0012B20f	5	14.6		NP_567479: *Arabidopsis* expressed protein
PP_LEa0010M17f	5	14.6		No match
PP_LEa0010K11f	5	14.6		No match
PP_LEa0010I18f	5	14.6		No match
PP_LEa0010C17f	5	14.6		No match
PP_LEa0009N08f	5	14.6		NP_195026: *Arabidopsis* calcineurin B-like protein 10 (CBL10)
PP_LEa0009L10f	5	14.6		No match
PP_LEa0010B17f	5	14.6		No match
PP_LEa0010C09f	5	14.6		NP_565558: *Arabidopsis* expressed protein
PP_LEa0009H20f	5	14.6		No match
PP_LEa0009G20f	5	14.6		NP_194405: *Arabidopsis* hydroxyproline-rich glycoprotein family protein
PP_LEa0009I06f	5	14.6		AAB88874: *Prunus armeniaca* enoyl-CoA hydratase
PP_LEa0008N06f	5	14.6		No match
PP_LEa0008L17f	5	14.6		No match
PP_LEa0009A05f	5	14.6		No match
PP_LEa0015O09f	5	19.7		NP_564132: *Arabidopsis* expressed protein
PP_LEa0012I12f	5	19.7		AAK85402: *Camellia sinensis* beta-1,3-glucanase
PP_LEa0011P02f	5	19.7		CAA11417: *Brassica juncea* ATP sulfurylase
PP_LEa0012C18f	5	22		NP_191593: *Arabidopsis* palmitoyl protein thioesterase family protein
PP_LEa0010I06f	5	46.3	Fruit skin pubescence	NP_193325: *Arabidopsis* phosphatases pleiotropic regulator 1 (PRL1)
PP_LEa0014B09f	5	46.3	Fruit skin pubescence	AAL67018: *Arabidopsis* putative elongation factor
PP_LEa0009A14f	5	46.3	Fruit skin pubescence	NP_187344: *Arabidopsis* transducin family protein
PP_LEa0009B20f	5	53.6		AAB99745: *Triticum aestivum* hsp70 protein
PP_LEa0011C13f	5	53.6		NP_196457: *Arabidopsis* expressed protein
PP_LEa0009B18f	5	53.6		AAM13199: *Arabidopsis* alpha-galactosidase-like protein
PP_LEa0013C06f	5	53.6		AAD22612: *Medicago sativa* subsp. x *varia* cell cycle switch protein
PP_LEa0009N05f	5	53.6		NP_568195: *Arabidopsis* ferredoxin-thioredoxin reductase, putative

EST Name	Linkage Group	Location (cM)	Trait association	Homology
PP_LEa0010P17f	6	0	Leaf shape	NP_192879: *Arabidopsis* Myb-like DNA-binding domain-containing protein
PP_LEa0011L02f	6	0	Leaf shape	No match
PP_LEa0013F16f	6	0	Leaf shape	NP_199101: *Arabidopsis* lipin family protein
PP_LEa0012K19f	6	0	Leaf shape	NP_564018: *Arabidopsis* oligouridylate-binding protein, putative
PP_LEa0010K20f	6	0	Leaf shape	NP_172261: *Arabidopsis* ribosomal protein L29 family protein
PP_LEa0009B20f	6	6.4	Dwarf plant	AAB99745: *Triticum aestivum* hsp70 protein
PP_LEa0011C13f	6	6.4	Dwarf plant	NP_196457: *Arabidopsis* expressed protein
PP_LEa0010P17f	6	12	Pollen sterility	NP_192879: *Arabidopsis* Myb-like DNA-binding domain-containing protein
PP_LEa0010P17f	6	17.5	Pollen sterility	NP_192879: *Arabidopsis* Myb-like DNA-binding domain-containing protein
PP_LEa0015M13f	6	20		NP_192880: *Arabidopsis* short-chain dehydrogenase/reductase (SDR) family protein
PP_LEa0015M20f	6	20		BAA81763: *Oryza sativa* putative transmembrane 9 superfamily protein member 2 precursor
PP_LEa0010P17f	6	28		NP_192879: *Arabidopsis* Myb-like DNA-binding domain-containing protein
PP_LEa0013A14f	6	39.3		AAK25976: *Arabidopsis* unknown protein
PP_LEa0013C06f	6	39.3		AAD22612: *Medicago sativa* subsp. x varia cell cycle switch protein
PP_LEa0010H23f	6	44.6		No match
PP_LEa0012K19f	6	46.4		NP_564018: *Arabidopsis* oligouridylate-binding protein, putative
PP_LEa0010H23f	6	79.6	Self-incompatibility (almond & apricot)	No match
PP_LEa0012K19f	6	85		NP_564018: *Arabidopsis* oligouridylate-binding protein, putative
PP_LEa0013H23f	6	86		No match
PP_LEa0009L10f	6	86		No match
PP_LEa0010B17f	6	86		No match
PP_LEa0016G11f	6	86		No match
PP_LEa0016A20f	6	86		No match
PP_LEa0013M13f	6	86		No match
PP_LEa0012O15f	6	86		No match
PP_LEa0012O21f	6	86		NP_177616: *Arabidopsis* nodulin family protein
PP_LEa0011F12f	6	86		No match
PP_LEa0012B20f	6	86		NP_567479: *Arabidopsis* expressed protein
PP_LEa0010M17f	6	86		No match
PP_LEa0010K11f	6	86		No match
PP_LEa0010I18f	6	86		No match
PP_LEa0010C17f	6	86		No match

EST Name	Linkage Group	Location (cM)	Trait association	Homology
PP_LEa0009N08f	6	86		NP_195026: *Arabidopsis* calcineurin B-like protein 10 (CBL10)
PP_LEa0010C09f	6	86		NP_565558: *Arabidopsis* expressed protein
PP_LEa0009G20f	6	86		NP_194405: *Arabidopsis* hydroxyproline-rich glycoprotein family protein
PP_LEa0009I06f	6	86		AAB88874: *Prunus armeniaca* enoyl-CoA hydratase
PP_LEa0008N06f	6	86		No match
PP_LEa0008L17f	6	86		No match
PP_LEa0009A05f	6	86		No match
PP_LEa0010G22f	7	34	Leaf gland shape	P05478: *Glycine max* hsp18.5 protein
PP_LEa0012L07f	7	36.7		No match
PP_LEa0013A21f	7	52.9		No match
PP_LEa0014B23f	7	59.5		AAL07042: *Arabidopsis* unknown protein
PP_LEa0013G17f	7	59.5		No match

[1]Location is approximate, as it is often inferred from the marker position from several maps.
[2]A peach ortholog for the sharka (plum pox virus) resistance locus is known to exist (A. Abbott, personal communication).