ABSTRACT

MCCORD, PER HILDING. Genetic, Genomic, and Transgenic Approaches to Understand Internal Heat Necrosis in Potato. (Under the direction of G. Craig Yencho and Bryon R. Sosinski.)

Internal heat necrosis (IHN) is a physiological disorder of potato tubers. A multifaceted approach was followed to better understand IHN. This approach entailed three major projects, involving genetic, genomic, and transgenic techniques.

The genetics approach involved developing linkage maps of tetraploid potato using AFLP and SSR markers, and identifying QTL for IHN. Our mapping population consisted of 160 individuals from a cross between 'Atlantic', an IHN-susceptible cultivar, and B1829-5, an IHN-resistant breeding line. Phenotypic data indicated that the distribution of IHN is highly skewed toward resistance. Early foliage maturity was positively correlated with reduced IHN. QTL for resistance to IHN were located in multiple years on chromosomes IV, V, and group VII of 'Atlantic', and on group VII of B1829-5. The QTL explained between 6.7 and 35.2 percent of the variation for mean severity, and from 4.9 to 32.5 percent of the variation for percent IHN incidence. Two IHN-linked markers from chromosomes I and V were also associated with IHN in a second population. The correlation between maturity and IHN may be partially explained by loosely linked QTL on chromosomes II and V. QTL were also detected in this population for tuber dry matter content, specific gravity, skin texture, yield, and flower color.

The genomic approach entailed a microarray-based gene expression analysis of 'Atlantic' potatoes grown under normal and high temperatures in growth chambers. Although overall levels of IHN were not significantly different between temperatures, IHN symptoms increased between the first and last harvest dates under high temperature. Mixed model analysis identified four genes whose expression was different between high and normal temperatures at the first and last harvest dates. Expression of one gene, F3C3.6, was

validated via quantitative RT-PCR (qRT-PCR). F3C.6 showed no difference in expression between harvest dates at high temperature, but was down-regulated over time under normal temperature. Quantitative RT-PCR of F3C3.6 using field-grown potatoes showed no differences between clean and IHN-affected tubers, supporting the hypothesis that down-regulation of this gene over time is associated with resistance to IHN.

Tuber calcium levels may be involved in the expression of IHN, and the transgenic project was designed to test this association. 'Atlantic' was transformed via *Agrobacterium tumefaciens* with a maize calreticulin-derived calcium binding peptide (*CBP*) shown to increase bioavailable calcium in *Arabidopsis*. Plants were grown under mild heat stress conditions in growth chambers. Transgenic lines generally had higher yields than wild-type plants. Levels of IHN in transgenic tubers were equal to or higher than wild-type tubers. Quantitative RT-PCR of *CBP* showed higher expression in one transgenic line, but also suggested that differing protein abundance underlies phenotypic differences between transgenic lines. Two native calreticulin genes were assayed by qRT-PCR for evidence of silencing, but none was found. Nutrient analysis of calcium and 11 other nutrients was also performed. Calcium levels in leaves of two transgenic lines were higher than wild-type 'Atlantic'. Leaf levels of magnesium, manganese, sulfur, and sodium were also higher than 'Atlantic' in at least one line. Most of these minerals are involved in photosynthesis; increased amounts in leaves could be responsible for the yield increase. In tubers, mineral levels were not significantly different. It is possible that expression of *CBP* in tubers is much lower than in leaves, which could explain the failure to detect any mineral differences. The increase in IHN could be due to the interruption of a signal from the leaves, or a subtle effect on tuber minerals that would require larger sample sizes to detect.

Genetic, Genomic, and Transgenic Approaches to Understand Internal Heat Necrosis in Potato

by
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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.

Per married Julie 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. Per's master's research involved physical mapping of peach fruit ESTs, and an initial search for DNA markers for internal heat necrosis in potato. He completed his M.S. in Horticulture in August 2005, and immediately began his Ph.D. research in potato genetics and breeding. Per and Julie are the parents of three sons, who all enjoy plants. In addition to plants, Per enjoys reading (mostly non-fiction), weightlifting, wrestling with his sons, playing the piano, fixing cars, watching movies with his wife, photography, and collecting firearms.

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I worked long and hard in order to complete this Ph.D., but, so did a LOT of other people. Without any doubt or qualification, my wife Julie is first on the list. Without her support, encouragement, patience, and understanding, graduate school would have been a lot more difficult and a lot less fun. I would also like to thank my three sons, Kurt, Ansel, and Lars, for helping me to have proper perspective in the middle of a hectic schedule.

A special acknowledgement goes to my committee members. Thank you, Craig, Bryon, Jason, and Niki! Your guidance, support, counsel, and refusal to panic made it possible for me to do this. I thank you, Craig, for the many conversations on long drives to and from Plymouth, and in your office, where you undoubtedly had many other things to do. Thanks for pushing me, and making a conscious effort to instill leadership. Bryon, thank you for always being willing to entertain my 'technical questions', helping me think outside the box I sometimes found myself in, and letting me tinker with expensive equipment. Jason, as Bill Murray once said, "we're just ropes on the Goodyear blimp" when it comes to your knowledge of statistics. (that line is from 'What about Bob?', in case you didn't know). I thank you for your patience as you helped me wade through the mountain of microarray data, and translated my questions and ideas into quantifiable tests and simulations. Niki, thank you for being calm and positive when I thought I would never get anywhere with my transgenic project, for freely offering me your insight and advice. Your confidence has boosted my own.

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INTRODUCTION

Internal heat necrosis, or IHN, is a serious physiological disorder of potato. A thorough review of IHN, discussing the symptoms, economic impact, and possible causes of the disorder was recently published in the American Journal of Potato Research (Yencho et al. 2008). IHN is characterized by necrotic patches in the parenchyma of the tuber, internal to the vascular ring (Larson and Albert 1945; Henninger et al. 1979). The necrotic patches typically 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).

Economic impact

Economic losses from IHN are extensive, and the disorder is a significant production constraint in the mid-Atlantic and southeastern United States, including Florida, North Carolina, Virginia, and New Jersey. Sterrett et al. (1991b) developed a 9-point rating system to express the severity of IHN symptoms, with 9 being completely free from IHN, and 1 being almost completely necrotic. Potatoes rated a 7 or lower are considered off-grade by USDA grading standards (Sterrett and Henninger 1997; USDA-AMS 2008). Sterrett and Wilson (1990) reported that from 3 to 11% of the acreage in North Carolina and Virginia was unharvested because plantings were off-grade due to IHN, and similar figures were reported later by Sterrett et al. (2006). Depending on yield, levels of IHN, and commodity prices, losses from IHN can easily amount to one million dollars (or more) per year in North Carolina alone (USDA-NASS 2009).

Environmental influences

Sterrett et al. (1991a, 1991b) and Lee et al. (1992) developed field-based predictive models for IHN using 'Atlantic' (Webb et al. 1978), a processing variety known for its susceptibility to the disorder (Henninger et al. 1979). Consistently, elevated temperatures, particularly in the early growing season (before 60 days after planting), were associated with increased levels of IHN. Increasing numbers of rain events (as opposed to increasing total rainfall), tended to decrease levels of IHN. Considerable research has been done on the relationship between calcium and IHN. Kelly and Christiansen (1970), Collier et al. (1978), and Olsen et al. (1996) all demonstrated a link between calcium and tuber necrosis under greenhouse conditions. Field studies have been less conclusive. Tzeng et al. (1986), Silva et al. (1991), Sterrett and Henninger (1991), and Clough et al. (1994) all demonstrated some association between increased soil and/or tuber calcium and IHN, but the results were inconsistent.

Genetics of IHN

Variation between cultivars for susceptibility to IHN has been observed for many years (Larson and Albert 1945, 1949; Ellison 1953; Wolcott and Ellis 1959; Sterrett and Henninger 1991). An enzymatic analysis of several free-radical scavenging enzymes by Davies and Talbot (1989) showed that 'Desiree', an IHN-resistant cultivar, had higher activities of these enzymes than an IHN-susceptible line, 10337de40. Broad-sense heritability for IHN is high, with estimates for percent incidence and mean severity of 0.83 and 0.85, respectively (Henninger et al. 2000). In addition, Sterrett et al. (2003) found resistance to IHN, as well as high specific gravity and yield, in 4x-2x hybrids of *Solanum tuberosum* X *S. phureja-stenotomum*. Aside from these more general observations, nothing more specific is known regarding the genetics of IHN.

With input from my dissertation committee, I designed a multi-faceted research project to better understand the environmental, nutritional, and genetic factors that contribute

to IHN and generate useful information for breeders. Information about IHN was obtained from experiments involving genetic, genomic, and transgenic approaches. This strategy is perhaps best illustrated using the Venn diagram shown below (Figure 1).

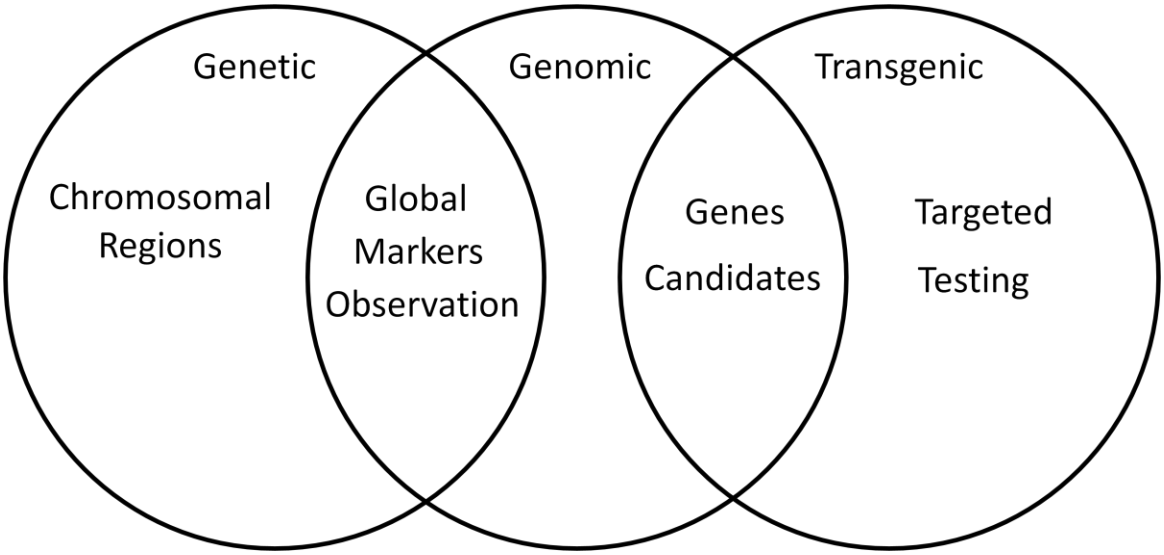

Figure 1. Venn diagram illustrating the similarities and differences between the genetic (QTL mapping), genomic (DNA microarrays), and transgenic portions of my Ph.D. research.

Genetic Aspects of IHN Resistance

Using AFLP and SSR markers, we developed a linkage map of tetraploid potato from a population segregating for IHN and other traits, and searched for QTL for these traits. We wanted to estimate the numbers, locations, and effects of QTL involved in IHN, as well as QTL for yield, specific gravity, percent dry matter, foliage maturity, and skin texture. We also wanted to identify individual molecular markers for testing relative to their use in marker-assisted breeding. A secondary mapping population also segregating for IHN was used to test several of these promising markers, in order to estimate the applicability of these markers for selection for IHN resistance in different crosses. This was a global,

observational approach, designed to scan the genome for chromosomal regions likely to be involved in traits of interest, and result in information for later, more targeted experiments such as testing of markers for selection, and fine mapping and characterization of QTL (Figure 1). This research is presented in chapters 1 and 2.

The cultivated potato is an autotetraploid (2n = 4x = 48), and therefore displays tetrasomic inheritance. Although linkage and QTL mapping is more easily performed at the diploid level, most of the breeding for commercial cultivars utilizes tetraploid materials. Disomic models of gene action may not be adequate to explain the behavior of loci in a tetrasomic context, because they do not account for the tri- and tetra-allelic interactions that can occur in tetraploids. Furthermore, polyploidy itself can induce changes in the organization of the genome (Song et al. 1995; Luo et al. 2004), and can affect gene expression (Lu et al. 2006; Pan 2008). These two phenomena could make it difficult to extrapolate mapping and QTL information from diploids. The difficulty of mapping in autotetraploids is lessened through the use of the software package TetraploidMap for Windows (Hackett et al. 2007), hereafter referred to as TetraploidMap. In particular, TetraploidMap can handle the segregation patterns of dominant markers in both simplex and duplex configurations, as well as co-dominant markers such as SSRs. TetraploidMap also includes algorithms for interval mapping of QTL. Therefore, this software package was used to conduct our mapping and QTL analyses.

Genomic Aspects of IHN Expression

Gene expression analysis using DNA microarrays can be used to screen many genes simultaneously, and identify candidate genes for a trait of interest (Salentjin et al. 2003; Lan et al. 2004). The genomic facet of this work entailed the use of DNA microarrays to analyze gene expression in potato tubers. Specifically, we analyzed gene expression in 'Atlantic' potato tubers during tuber bulking, and under elevated and non-elevated temperatures. We utilized a spotted cDNA microarray developed by The Institute for Genome Research (TIGR,

now part of the J. Craig Venter Institute, www.jcvi.org), that contains over 11,400 unique potato genes. This array has been used to identify candidate genes for drought tolerance (Schafleitner et al. 2007), and defense responses to feeding by Colorado potato beetle, *Leptinotarsa declemineata* (Say) (Lawrence et al. 2008). The results of this experiment were expected to explore the influences of time and temperature on the development of IHN under controlled conditions, and identify candidate genes that are differentially expressed between treatment regimes with varying levels of heat necrosis. These candidate genes could then be used as a basis for hypothesis-driven research into the biology of the disorder, or screened for polymorphism which could be used in marker-assisted breeding. As with the genetic component of our work, the microarray experiment was a global approach, in this case analyzing expression levels of specific *genes* located across the genome, and an observational study designed to guide us toward later, more targeted experiments. Chapter 3 details our microarray experiment and its results.

Transgenic Aspects of IHN Resistance

One of the most robust ways to determine if a specific gene(s) contributes to a phenotype is to transform a plant with a gene of interest (a candidate gene), and compare the phenotypes of wild-type and transformed plants. In potato, for example, the *RB* gene from *S. bulbocastanum* contributes broad spectrum resistance to late blight, *Phytophthora infestans*. A candidate *RB* gene was isolated using map-based cloning and long-range PCR, but the proof that the isolated sequence was indeed *RB* was provided by transforming a susceptible cultivar ('Katahdin') with the putative *RB* gene, and demonstrating resistance to late blight (Song et al. 2003). To specifically address the calcium hypothesis vis-à-vis IHN, we followed a transgenic approach, described in chapter 4. Wyatt et al. (2002) and Lee et al. (in preparation) have shown that expression in *Arabidopsis* of a maize calreticulin-derived calcium binding peptide (*CBP*) increased bioavailable calcium under calcium-deficient conditions, and increased tolerance to drought and salt stress. Using the same *CBP* under the

control of a constitutive promoter (CaMV 35S), we transformed 'Atlantic' potato. Our objectives were designed to address two questions. First, would 35S:*CBP* increase calcium levels in potato tubers? Second, would a calcium increase in tubers result in lower levels of IHN? As with the genomic research project, the transgenic approach dealt with an actual gene rather than an approximate chromosomal region. In contrast to the genetic and genomic approaches, the third project was a targeted experiment, designed to directly test the relationship between calcium and IHN under controlled conditions.

Conclusion

The Ph.D. research described in the succeeding chapters follows the multi-part strategy explained in this introduction. Through the use of QTL mapping (genetic), DNA microarrays (genomic), and transgenic techniques, we expected to generate significant new knowledge regarding internal heat necrosis of potato. This included: 1. Estimates of the numbers of loci involved in the trait and their effects, and a set of markers linked to these loci that could be used for further testing; 2. A group of candidate genes for IHN that could then be screened for markers for use in selection, or for further, more targeted experiments; 3. Clearer demonstration of the relationship (or lack thereof) between calcium and IHN. Accomplishment of these objectives will both increase the body of knowledge of IHN, and prepare the way for future research into this important disorder of potato.

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Chapter One

QTL Mapping of Internal Heat Necrosis (IHN) in Tetraploid Potato

Per H. McCord, Bryon R. Sosinski, Kathleen G. Haynes, Mark E. Clough, and G. Craig Yencho

QTL mapping of internal heat necrosis (IHN) in tetraploid potato

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Abstract

Internal heat necrosis (IHN) is a serious physiological disorder of potato (*Solanum tuberosum* L.) tubers. Aside from heritability estimates, relatively little genetic research has been conducted to understand the trait and develop tools for breeding for IHN resistance. We developed a linkage map of tetraploid potato using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, and used interval mapping to detect quantitative trait loci (QTL) for mean IHN severity and percent IHN incidence. Phenotypic data collected over three years indicated that the distribution of IHN is highly skewed toward resistance, even in a cross between susceptible and resistant parents. Early foliage maturity was slightly but significantly correlated with reduced IHN symptoms. The linkage map for 'Atlantic', the IHN-susceptible parent, was 1051.8 cM in length and contained 13 linkage groups, of which eight were anchored to known *Solanum* chromosomes through the use of mapped SSR markers. The map for B1829-5, the IHN-resistant parent, covered 957.7 cM and contained 14 linkage groups, of which nine were anchored. QTL for increased resistance to IHN were located on chromosomes IV, V, and group VII of the 'Atlantic' map and on group VII in the B1829-5 map in at least two of three years. The QTL explained between 6.7 and 35.2 percent of the variation for mean severity, and from 4.9 to 32.5 percent of the variation for percent IHN incidence. As suggested by the skewed phenotypic distribution, most QTL detected were associated with decreased IHN symptoms, and had dominant effects.

One SSR and 13 AFLP markers that were linked to IHN were tested in a secondary population. One AFLP marker, found on chromosome I of B1829-5 and associated with decreased IHN symptoms, was also associated with decreased symptoms in the secondary population. The SSR marker, from chromosome V, was not associated with IHN in the second population, but was closely linked (2.9 cM) in repulsion to another marker that was associated with IHN, and had the same negative effect on the trait as the SSR marker did in the primary population. The correlation between maturity and IHN may be partially

explained by the presence of a QTL for maturity on chromosome V, loosely linked to the QTL for IHN. This research represents the first major molecular genetics study of IHN in potato. More simplex and multi-allelic markers are needed to provide better genome coverage to detect QTL for IHN resistance, and to model non-dominant and epistatic effects before marker-assisted breeding for IHN resistance in potato is implemented, but this work represents a valuable first step.

Introduction

Internal heat necrosis, or IHN, is a significant physiological disorder of potato tubers. It is characterized by necrotic patches in the parenchyma of the tuber, internal to the vascular ring (Larson and Albert 1945; Henninger et al. 1979). The necrotic patches typically 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).

Economic losses from IHN are extensive, and the disorder is a significant production constraint in the mid-Atlantic and southeastern United States, including Florida, North Carolina, Virginia, and New Jersey. Sterrett et al. (1991a) developed a 9-point rating system to express the severity of IHN symptoms, with 9 being completely free from IHN, and 1 being almost completely necrotic. Potatoes rated a 7 or lower are considered off-grade by USDA grading standards (Sterrett and Henninger 1997; USDA-AMS 2008). Sterrett and Wilson (1990) reported that as much as 11% of the potato acreage in North Carolina is left unharvested due to levels of IHN causing plantings to go off-grade. A major reason for these losses is because 'Atlantic', a chipping cultivar grown on a majority of commercial acreage in the aforementioned regions, is highly susceptible to IHN (Webb et al. 1978; Henninger et al. 1979).

A review of methods to control IHN (Sterrett and Henninger 1997) concluded that the most efficacious option would be to breed for IHN resistance. Although broad-sense heritability for IHN resistance is high (Henninger et al. 2000), breeding for resistance is difficult. This is because the intensity of symptoms can vary from year to year due to the strong influence of environment on IHN expression, requiring genotype evaluations over several years and locations (Yencho et al. 2008, P. McCord, personal observation). In

addition, there are no above-ground or external symptoms of IHN, necessitating that tubers be harvested and cut in order to observe the phenotype. Finally, IHN resistance/susceptibility is quantitative (reviewed in Yencho et al. 2008), making it more difficult to predict the behavior of individual genotypes. All of these situations increase the time and expense involved in breeding for IHN resistance. The development of molecular markers linked to IHN would greatly facilitate breeding by reducing the amount of land and time required to screen for the trait. When these markers are identified in the course of developing a genetic linkage map based on a segregating population, important information is also gained regarding the genetics of the trait, i.e. the number, effect and chromosomal location of genes and/or QTL involved.

Our objectives for this research were to generate a genetic linkage map and identify QTL for IHN resistance/susceptibility in a segregating population of tetraploid potato. Although linkage and QTL mapping is more easily performed at the diploid level, most of the breeding for commercial cultivars utilizes tetraploid materials. Disomic models of gene action may not be adequate to explain the behavior of loci in a tetrasomic context, because they do not account for the tri- and tetra-allelic interactions that can occur in tetraploids. Furthermore, polyploidy itself can induce changes in the organization of the genome and changes in gene expression, making it difficult to extrapolate map information from diploids (Song et al. 1995; Luo et al. 2004). The difficulty of mapping in autotetraploids is lessened through the use of the software package TetraploidMap for Windows (Hackett et al. 2007), hereafter referred to as TetraploidMap. In particular, TetraploidMap can handle the segregation patterns of SSRs and other multi-locus markers, and includes algorithms for interval mapping of QTL. We used AFLP markers (Vos et al. 1995) for the bulk of our marker coverage, and include a small number of previously mapped SSR markers to anchor our linkage groups to known *Solanum* chromosomes. We also tested the utility of markers associated with QTL in a second tetraploid population.

Materials and Methods

Populations

Population B2721, the primary mapping population, consisted of 160 individuals from the cross 'Atlantic' X B1829-5. 'Atlantic', a processing cultivar released in 1978, (Webb et al. 1978) is highly susceptible to IHN. B1829-5, a breeding line from the USDA-ARS Beltsville potato breeding program, is highly resistant. Progeny (20 genotypes) from this cross were first observed in 2004 and 2005 as part of a larger study of general combining ability for heat necrosis (Haynes, unpublished), and were found to be segregating for the trait. During 2005, minitubers generated from remnant seed from this cross were grown in the field as single plants. The first 160 plants yielding enough tubers for at least 10 plants per genotype for the following year were collected to form the B2721 mapping population. Population NC206, the population used for testing B2721-derived markers, consisted of 163 individuals from the cross B2464-1 (IHN-susceptible) X B2471-5 (IHN-resistant). Both parents were identified in the general combining ability study mentioned earlier. In addition, both parents were derived from crosses between 4x *Solanum tuberosum* X 4x-2x *S. phureja-stenotomum*. These diploid species are a source of high dry matter and reduced susceptibility to IHN (Sterrett et al. 2003). In 2006, 282 individuals from this cross producing more than one tuber as seedlings were grown as single plants at the USDA-ARS Chapman Farm, Presque Isle, Maine for seed increase (250 of this group were also planted in the greenhouse in North Carolina for data collection). Of the individuals planted in Maine, 163 produced enough tubers for at least 9 plants, and these constituted population NC206.

Field experimental design and data collection

B2721 was grown in 2006 using an unreplicated design with 6 plants per plot. In 2007 and 2008, the population was planted in a randomized complete block design with 2 replications and 10 plants per plot. NC206 was grown in 2007 as an unreplicated design with 4 plants per plot, and in 2008 was grown as for B2721. All field trials were conducted at the

North Carolina Department of Agriculture and Consumer Services, Tidewater Research Station (NCDA&CS TRS) in Plymouth, North Carolina, in a Portsmouth fine sandy loam. Fertilizer was applied at planting (18-18-18, 772.9 kg/ha and 30-0-0, 64.9 L/'ha) and at hilling (30-0-0, 205 L/ha). Seed pieces were not treated prior to planting. Insect control was provided (depending on year) by carbamate, imidacloprid, pyrethroid/nicotinoid (Leverage®), and spinosad using recommended rates. Early season weed control was provided by pre-emergent treatment with s-metolachlor and metribuzin; middle and late season weeding was done by hand.

Four and six-plant plots were dug with a single-row chain digger and harvested by hand. Ten-plant plots were harvested with a two-row potato harvester, modified to dig one row. Tubers were then washed and culled for rot only. For evaluation of IHN, tubers from each plot were quartered longitudinally, and the most affected quarter was retained for scoring. In 2006, a minimum of 40 tubers were cut per plot. If any of the first 40 tubers had IHN symptoms, all remaining tubers of marketable size were cut as well. In 2007 and 2008, 20 tubers were cut per plot. Due to lower rainfall and subsequent lower yields, 20 tubers were not always available in 2007 and 2008. In these cases, we cut all marketable tubers. Two measures of IHN were employed; severity and incidence. Severity refers to the mean severity rating of all tubers examined in a plot, based on a 9-point rating system developed by Sterrett and Henninger (1997), with 9 representing no symptoms and 1 representing completely necrotic tubers, respectively. Incidence was determined by calculating the percentage of sampled tubers displaying any level of IHN. Both populations were also rated for yield (on a per-plant basis), foliage maturity, skin texture, specific gravity, dry matter, and flower color (McCord et al. in preparation). In addition, NC206 was rated for flesh color. Variation of IHN symptoms due to year and population, and correlation between IHN and other measured traits was explored using the MIXED and CORR procedures, respectively, of SAS (SAS, Cary, NC). Years were treated as random when testing for population effects, and fixed when testing for year and year-by-population effects.

DNA extraction and marker generation

DNA for AFLP and SSR analysis was extracted primarily from greenhouse-grown leaf tissue (in three cases, DNA extractions were done using sprouts and surrounding tissue from tubers). Leaf tissue was placed in a microcentrifuge tube, which was then briefly dipped (not immersed) in liquid nitrogen. Tissue samples were then ground manually using miniature plastic pestles. After addition of CTAB extraction buffer (www.cipotato.org/csd/materials/Molecular/Molecular1.pdf), samples were incubated in a water bath at 65º C for 30-60 minutes. Following extraction with 24:1 chloroform:isoamyl alcohol, nucleic acids were precipitated with isopropranol and concentrated by centrifugation. Pellets were washed with 70% ethanol, dried, and resuspended in buffer (10 mM Tris, 0.1 mM EDTA pH 8) supplemented with RNase A (approximately 130 µg/mL final concentration).

Templates for AFLP fingerprinting with *Eco*RI and *Mse*I were prepared essentially as described in Myburg and Remington (2000), which is a version of the original protocol of Vos et al. (1995) modified to use fluorescence-based fragment detection. For fingerprinting with *Pst*I and *Mse*I, the second-digest AFLP (SD-AFLP) method was used, essentially following the method developed by Knox and Ellis (2001). The SD-AFLP method employs initial restriction, ligation, and preamplification reactions using only *Mse*I enzyme, adapters, and primers. This results in template DNA that is free of methylated cytosine residues (5-methylcytosine) to which *Pst*I is sensitive (McClelland et al. 1994 and references therein). Following restriction and ligation with *Pst*I enzyme and adapters, the SD-AFLP procedure is identical to the AFLP method. Preamplification reactions (20 µL) consisted of 1X PCR buffer (10mM Tris-HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X 100), 800 µM (total) dNTPs, 30 ng each of *Eco*RI (or *Pst*I) and *Mse*I preamplification primers, 1.2 units *Taq* polymerase, and 5 µL of a 1:10 dilution of the restriction/ligation reaction. PCRs were performed in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer, Waltham, MA) as follows: 28 cycles of 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute,

followed by a final incubation at 72°C for 2 minutes, and an indefinite hold at 4°C. Selective amplifications for all enzyme combinations were performed in 10 µL volumes consisting of 1X PCR buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl, 1.5mM $MgCl_2$, 0.1% Triton-X 100), 800 µM (total) dNTPs, 0.5 pmol forward (*Eco*RI or *Pst*I-targeted) primer, 15 ng reverse (*Mse*I-targeted) primer, 0.6 units *Taq* polymerase, and 2 µL diluted preamplified template DNA. PCR cycling conditions were as follows: 15 cycles of 94°C for 30 seconds, 65°C for 30 seconds, decreasing 0.7° each cycle, and 72°C for 1 minute: 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute: 72°C for 2 minutes to complete extension, followed by an indefinite hold at 4°C. *Eco*RI and *Pst*I primers were labeled at the 5'end with either IRD-800 or IRD-700 fluorescent dyes. Reaction products were separated and visualized using Li-Cor 4000, 4000L, and 4300 DNA analyzers (Li-Cor, Lincoln, NE).

SSR markers were selected from marker lists published by Frary et al. (2005) for tomato, and Feingold et al. (2005) for potato. Markers were screened for polymorphism in B2721 via electrophoresis in agarose or Spreadex® EL 800 (Elchrom Scientific, New Hyde Park, NY) gels, utilizing the parents and ten individuals. Two SSRs (SSR46 and SSR73) were analyzed across the entire population using agarose gel electrophoresis. The PCR reactions for SSR46 and SSR73 were performed in 10 µL volumes consisting of 1X PCR buffer, 800 µM (total) dNTPs, 300 nM forward and reverse primers, 0.6 units *Taq* polymerase, and 2 µL genomic DNA (generally diluted 1:10 from original extraction, in 10mM Tris, 0.1mM EDTA pH 8). Reaction conditions were as follows: 94°C for 2 minutes 30 seconds, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds (90 seconds for SSR73), followed by a final extension at 72°C for 7 minutes and an indefinite hold at 4°C. The remaining SSR markers were amplified using a 3-primer M13 'tail' protocol (Schuelke 2000, Rampling et al. 2001), and analyzed using the Li-Cor systems as for AFLP. PCR reactions were performed in 10 µL reaction volumes consisting of 1X PCR buffer, 800 µM (total) dNTPs, 0.1 pmol forward primer with a 5' M13 tail, 0.5 pmol reverse primer, 0.5 pmol M13 primer (5' CACGACGTTGTAAAACGAC 3') labeled

with IRD-800 or IRD-700, 0.6 units *Taq* polymerase, and 2 µL genomic DNA. Cycling conditions were as follows: 15 cycles of 94° C for 30 seconds, 65°C for 30 seconds, decreasing 1° each cycle, and 72°C for 1 minute: 25 cycles of 94° C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute: 72°C for 7 minutes to complete extension followed by an indefinite hold at 4°C. For all markers, gel images were adjusted for ease of scoring and converted to JPEG format using Adobe Photoshop (Adobe Systems Inc., San Jose, CA), FastStone Image Viewer (www.faststone.org), or GIMP (www.gimp.org). Markers were then scored using the program Cross Checker (Buntjer 2000). Some SSR primers resulted in banding patterns that were difficult to score in a codominant fashion. In these cases, individual bands were scored as dominant markers. Information was lost regarding the locus *per se*, but this strategy still allowed us to anchor linkage groups to *Solanum* chromosomes.

Map construction and QTL analysis

For primary map construction and QTL analysis, we used the program TetraploidMap for Windows (Hackett et al. 2007). This software package is designed to infer parental genotype from the marker phenotypes of the parents and segregating progeny of a cross in an autotetraploid species. TetraploidMap for Windows and its predecessor TetraploidMap have been used to construct linkage maps in alfalfa (Julier et al. 2003; Robins et al. 2008) and potato (Bradshaw et al. 2004, 2008; Sagredo et al. 2006; Khu et al. 2008; Kelley et al. 2009). Once parental genotypes are determined at each locus (based on the marker phenotype or observed bands, and segregation ratios in the progeny), TetraploidMap attempts to combine the markers into linkage groups via cluster analysis, and determine the proper marker order. The software will accommodate dominant (AFLP) markers in simplex, duplex, or double-simplex configuration, and codominant (SSR, CAPS, and SCAR) markers, including any null alleles of codominant markers.

Based on experience using TetraploidMap, we selected the following types of markers for map construction: 1) simplex dominant markers (segregating 1:1) with a p-value

greater than 0.001 from a chi-square test for goodness-of-fit; 2) duplex dominant markers (segregating 5:1) with a p-value greater than 0.01; 3) double simplex dominant markers (segregating 3:1) with a p-value generally greater than 0.01 *and* which were known to be linked to at least one simplex marker; 4) all SSR (codominant) markers. Markers with greater than 10% of individuals missing were omitted. Following initial clustering of markers, the final linkage group composition and marker order were determined.

TetraploidMap offers several strategies for marker ordering. In most cases the 'ripple' option was used due to its combination of speed and accuracy. Simulated annealing (Hackett et al. 2003) can give more accurate ordering results, but takes a long time to compute; it was generally used only for groups with fewer than 20 markers. Homologous chromosomes/linkage groups were identified via simplex markers linked in repulsion (detected automatically in TetraploidMap), duplex markers, and SSRs. Double-simplex markers, which are present in both parents but segregate in their progeny, were used along with SSR markers to align the parental maps.

TetraploidMap also includes an interval mapping (IM) routine for QTL analysis. This IM routine utilizes a multi-step procedure. First, a graphical reconstruction is made of the chromosome/linkage group for each individual in the population, based on the parental map (the software considers data generated from only one parent at a time). In this first step, a branch and bound algorithm is used to create an individual's linkage group based on the fewest number of recombinations required to produce the observed segregation patterns (Hackett et al.2001). Second, conditional probabilities of each possible QTL genotype (at 2 cM intervals) are calculated based on the chromosome reconstructions. Finally, phenotypic values for each trait are regressed on putative QTL genotypes, each genotype being weighted by its conditional probability (Hackett et al.2001). The 'full' model includes six QTL genotypes, reflecting the six combinations in which any two parental alleles can be transmitted to offspring. Ten simpler models, which reflect a dominant effect of the QTL,

are also analyzed and compared to the full model by a likelihood ratio test. These include the six duplex genotypes tested individually, and the four simplex genotypes.

We utilized the IM routine of TetraploidMap for almost all linkage groups identified. We performed permutation tests (Churchill and Doerge 1994) of 100 iterations generally for all LOD scores greater than or equal to 2.5. LOD scores were also tested which displayed a clear peak, but were slightly less than 2.5. In cases where the LOD peak was between the 90 and 95th percentile of the test distribution, the permutation test was rerun at least once more before accepting or rejecting the presence of a QTL at that position. LOD scores below the 90 percent distribution point were rejected as evidence for a QTL, except in cases where the LOD score was at least 1.8, and was also significant in a different year according to the more stringent criteria (and within 10 cM of the LOD score being considered). Traits were analyzed separately for each year. When a simpler model was not significantly different, and passed the permutation tests, we reported the simpler model. Empirically, we determined that p-values for goodness of fit above 0.05 but below ~0.2 often did not pass the permutation test, while the full model did. In these cases, we reported the full model.

We found it was difficult to successfully perform the IM routine when analyzing a group with only one homolog. In these cases, we analyzed the marker class means using single-point ANOVA. Three linkage groups of B1829-5 (chromosomes I and XI and group XII) contained more than one homologous group, but could not be reliably analyzed via the IM routine of TetraploidMap. These groups were also tested for QTL using single-marker ANOVA. Only markers with associated p-values <0.01 were reported, except in cases (as with interval mapping) where the marker was significant for the trait of interest in at least one other year. In these cases, markers with p-values < 0.05 were reported as well. Occasionally, interval mapping generated significant LOD scores, despite the fact that no significant single markers (at $p < 0.05$) were present in the linkage group or were more than 35 cM from the LOD maximum; these QTL were considered spurious, and are not included in the results.

Results

Phenotypic data

Across all years, and both populations, the distributions of IHN incidence and severity were similar (Figures 1A-1E). They were all highly skewed, with most individuals displaying little to no IHN symptoms. Some transgressive segregation was observed, i.e. a handful of individuals were more susceptible to IHN than their susceptible parent. There were significant year effects on the population mean IHN susceptibility (p-values < 0.0001 for incidence and severity), but population means were not significantly different from each other ($F = 0.95$, p-value = 0.33), nor was there a population by year interaction ($F= 0.31$, p-value = 0.58). However, a number of *individuals* in both populations showed widely varying IHN symptoms from year to year (data not shown). For example, 'Atlantic' had an average IHN incidence of 59%, 15%, and 20% for 2006, 2007 and 2008, respectively. This is in agreement with Henninger et al.(2000), who determined that 'Atlantic' is unstable in its expression of IHN symptoms, even when environmental variation is taken into account. One of the members of B2721, B2721-87, had an average incidence of 0%, 35%, and 0% over the three seasons.

In population B2721, IHN incidence and severity were highly negatively correlated ($r = -0.95$, Table 1A), indicating that clones with a high percentage of IHN-affected tubers also have a low (more severe) severity rating. Correlations between IHN incidence or severity and other agronomic traits were either statistically insignificant, or had significant correlations of low magnitude ($r < 0.3$). The single exception was foliage maturity. Maturity was calculated as the area under the curve of a series of subjective visual assessments of foliage and vine senescence (McCord et al. in preparation). Therefore, lower maturity ratings indicate later maturity, and vice versa. Earlier maturity was negatively correlated with IHN incidence ($r = -0.44$), and positively correlated with IHN severity ($r = 0.34$).

IHN severity and incidence were also highly negatively correlated in NC206 (r = -0.94, Table 1B). As with B2721, earlier foliage maturity was also correlated negatively with incidence and positively with severity, though the coefficients were slightly lower (r = -0.357 and 0.284, respectively). In contrast to B2721, yield in NC206 was significantly correlated with IHN (r = 0.455 and -0.381 for incidence and severity, respectively).

Linkage maps

Forty *Eco*RI/*Mse*I primer combinations, seven *Pst*I/*Mse*I primer combinations, two SCARs, and 14 SSRs were analyzed in B2721, resulting in 674 scored markers. The AFLP primer combinations, and the number of markers generated per combination, are listed in Table 2. The parental genotypes of 24 markers could not be reliably determined from their segregation ratios, and were not used. Following the marker selection criteria described in the Materials and Methods section, subsets of the marker pool were used to create a linkage map for each parent.

The map for 'Atlantic' included 287 markers, of which 180 were simplex, 30 were duplex, 68 were double-simplex, and nine were SSRs, four of which were scored as dominant markers. The markers were resolved into 18 linkage groups. In several cases two or more groups were combined based on common linkage (via double-simplex markers) to a single group from B1829-5, resulting in a total of 13 linkage groups for 'Atlantic'. Ten of these groups contained all four homologs, and the remaining three (chromosome VIII, group XIII, and group XIV) contained one homolog each. Chromosomes I, II, III, IV, V, VI, VIII, and IX were anchored through the use of SSR markers, either directly in the 'Atlantic' map, or through the linkage of double-simplex markers to SSRs mapped in B1829-5. Thirty-two markers remained unlinked (this includes unanchored linkage groups with only two markers).

A total of 254 markers were used to generate the map for B1829-5. These included 144 simplex markers, 26 duplex markers, 74 double-simplex markers, and 10 SSRs (five scored as dominant). This dataset was organized initially into 16 linkage groups, later

condensed to 14 groups when some were combined via common association to linkage groups from 'Atlantic'. Five of these groups (chromosomes II, III, VI, IX, and group VII) contained all four homologs. Three homologs were identified in each of chromosomes I, IV, and V, two homologs each in chromosomes VIII, XI, and group XII. The remaining three groups (X, XIII, and XV) each consisted of a single homolog. Chromosomes I, II, III, IV, V, VI, VIII, IX, and XI were anchored via the use of previously mapped SSR markers. Twenty-five markers remained unlinked. The data in Figure 2 are presented as overall maps, with data from all homologs collapsed into one group as done by Bradshaw et al. (2008). Using double-simplex and microsatellite markers, alignment of linkage groups from parental maps was achieved for all but one group from each parent (group XIV from 'Atlantic', and group XV from B1829-5). Overall map lengths were 1051.8 cM for 'Atlantic', and 957.7 cM for B1829-5.

A high degree of marker clustering, presumably around the centromere, can be seen in many of the linkage groups (for example, chromosome V). Clustering of AFLP markers around the centromere has been reported previously (van Eck et al. 1995). In an attempt to broaden marker coverage, we complemented our *Eco*RI/*Mse*I –derived set of AFLP markers with approximately 100 additional *Pst*I/*Mse*I SD-AFLP markers. Markers derived from this enzyme combination are also known to cluster, but in different areas of the genome relative to *Eco*RI/*Mse*I –derived markers (van Os et al. 2006). Marker coverage was improved slightly, but it is difficult to determine if this was due to the new enzyme combination or simply the addition of more markers.

Detected QTL and significant single markers are shown graphically in Figure 1. As maturity was the only other trait correlated with IHN, only maturity, IHN severity, and IHN incidence QTL and/or markers are shown on the map.

IHN Severity

In 'Atlantic', nine QTL for IHN severity were detected via interval mapping (Table 3). Three QTL were identified in roughly the same position on chromosome IV, one for each year; the variation accounted by these three QTL ranged from 7.6% in 2006, to 35.2% in 2008. Four QTL (two in 2006, one in both 2007 and 2008) were also identified on chromosome V, though their position was more variable than those detected on chromosome IV. These loci explained from 7.5 to 9.3 percent of the observed variation, except for the second QTL found in 2007. Due to the constraints of interval mapping, which assumes there is only one QTL per linkage group corresponding to the maximum LOD score, the effects of the second QTL were not estimable. On linkage group VII, one QTL was identified in 2007 explaining 29.4% of the variation, and on linkage group X, one QTL was identified in 2006 that explained 10.4% of the variation. Of the eight QTL that could be modeled for dosage and effect, six were modeled as simplex or duplex alleles with dominant effect, while the remaining two QTL were best explained using the original 6-mean additive model. In each of these first six cases, the QTL was associated with decreased IHN severity (i.e. a higher score). For the QTL with additive effects (chromosome V in 2006 and 2008), QTL alleles from homologs 1 and 3 showed partial dominance for reduced severity over alleles from homologs 2 and 4, but the dominance was not complete enough to use a duplex dominant model to fit the data.

Five QTL for severity were identified in B1829-5 (Table 3). Three of these were found in a similar location (~34 cM) on linkage group VII (one in each year). The effects of the QTL from 2006 could not be estimated, as an additional QTL with higher LOD score (at 20 cM) was also detected that year. The QTL from 2007 and 2008 explained 8.5 and 7.7 percent of the variation, respectively. The QTL from 2007 was modeled as a duplex dominant allele for decreased severity, as was the QTL at 20 cM from 2006. One QTL was detected for severity in 2007 on chromosome V that explained 6.7 percent of observed variation.

A number of single markers in B1829-5 were associated with IHN severity, as revealed by single-point ANOVA (Table 4). On chromosome I, five linked markers were strongly associated with reduced severity in at least one year. One marker, E41M50_162.3, was detected in all three years. This simplex marker was highly skewed (p-value from chi-square test = 0.0007), but was retained because of its significance. Two markers on chromosome XI were associated with increased IHN severity in 2007 and 2008.

IHN Incidence

As expected from the high correlation between IHN incidence and severity, the QTL detected for incidence both through IM and single-point ANOVA were generally collinear with those for IHN severity. Eight QTL for IHN incidence were detected in 'Atlantic', of which seven were estimable with respect to dosage and effect (Table 3). Six of these eight QTL for IHN incidence were collinear with those for IHN severity. Again, most had dominant effects, and all dominant QTL were associated with decreased incidence of IHN. Variation explained by these QTL ranged from 6.8 to 32.5 percent. Five QTL (four estimable) were detected in B1829-5; three were collinear with QTL for IHN severity. The two QTL with dominant effects, on group VII, were detected in 2006 and 2008, and were associated with decreased IHN incidence. These QTL explained from 6.1 to 8.6 percent of the variation.

Five markers from chromosome I were associated with reduced incidence in at least one year, as was a single marker from group XII. Two markers from chromosome XI (including an SSR, SSR46 from tomato) were associated with increased incidence in 2007 and 2008. All of these markers were present in B1829-5.

In summary, based on observation over three years, we have reliably detected QTL for IHN on chromosomes IV and V and linkage groups VII and X, the majority of which exhibit dominant action. Based on single-marker ANOVA, there also appear to be QTL on chromosomes I and XI, and possibly on linkage group XII.

Maturity

QTL for foliage maturity were detected on 'Atlantic' chromosomes II, III, and V in both 2007 and 2008, and linkage group XII in 2007 (Figure 1 and Table 3). All but one of the QTL had dominant effects. The QTL from chromosomes II, V, and group XII were associated with later maturity (lower AUSC scores), while the QTL from chromosome III, at least in 2008, was associated with earlier maturity (higher AUSC scores). The variation explained by these QTL ranged from 5.3 to 14.3 percent. A single marker on chromosome IX, E45M58_101.7 was associated with later maturity in 2007. In B1829-5, a QTL was found in the same location on chromosome V in both 2007 and 2008, where it explained 9.6 and 15.6 percent of the phenotypic variation, respectively. This QTL exhibited dominant action, and was associated with earlier maturity. One marker each was found on chromosome I, group X, and group XII. These markers were significant in 2007 and 2008, and all were associated with later foliage maturity.

Marker testing in NC206

Four AFLP primer combinations (all *Eco*RI/*Mse*I) and one SSR marker, STI032_4 (the fourth largest allele of the SSR, scored as a dominant marker) were used to screen population NC206. One primer combination, E38M58, was chosen because it produced a marker shown to be associated with incidence and severity. STI032_4 was chosen because it was associated with increased IHN symptoms (both severity and incidence) in 2007 and 2008, and was linked to QTL for IHN incidence and severity on chromosome V. Primer combination E36M57 was chosen because it produced markers associated with yield, texture, and flower color (McCord et al. in preparation). The remaining primer combinations (E32M40 and E36M32) had already been used to develop markers in NC206 in an initial attempt to generate a second set of linkage maps. In B2721, the primer combinations produced 13 IHN-associated markers, on chromosomes I, III, VI, and group XII; two markers were unlinked. In NC206, the AFLP primer combinations produced 63 markers,

which were tested for their association with IHN, and their relationship (i.e. molecular weight) to the original B2721 markers. Eight of the original B2721 markers were polymorphic in NC206. One marker, E38M58_135, was strongly associated with reduced IHN severity and incidence in 2008 (Table 4). This marker is located on chromosome I of B1829-5, and was significantly associated with reduced incidence and severity over several seasons in this population as well (see Figure 1 and Table 3). In contrast to B2721, where the marker was contributed by the resistant parent, in NC206 the marker was contributed by B2464-1, the susceptible parent. STI032_4 was not associated with IHN *per se* in NC206, but it was in repulsion-phase linkage (i.e. on a different homolog) at a distance of 2.9 cM to an AFLP marker, E32M40_180.8, that was itself associated with increased IHN symptoms (severity and incidence) in both 2007 and 2008 (data not shown). STI032_4 was associated with increased IHN symptoms in B2721 as well, so we believe we have identified the same QTL in both populations. In the case of E32M40_180.8, the marker was contributed by the susceptible parent.

Discussion

QTL for IHN

This paper presents the first attempt to map and characterize QTL responsible for internal heat necrosis. The population distributions of IHN severity and incidence lead us to hypothesize that these two traits were controlled by relatively few genes of major effect. Skewed phenotypic distributions caused by the segregation of a major gene or genes have been demonstrated in other species, including *Mimulus* (flower carotenoids, Bradshaw et al. 1995), chickpea (ascochyta blight resistance, Cho et al. 2004), and citrus (citrus tristeza virus (CTV) resistance, Asins et al. 2004). The QTL for IHN detected in our research explained up to 35.2 and 32.5 percent of the variation for severity and incidence, respectively. These figures are not as large as for other major QTL, (for example, Bradshaw et al. (1995) detected a QTL which explained 88.3 percent of the variation for flower carotenoids), but

they still account for a sizable portion of the phenotypic variation. When the variation explained in a given year was summed over all QTL for each measure of IHN, they explained an average of 58 percent of the variation for severity, and 41.6 percent of the variation for incidence. In addition, the majority of the QTL we detected—15 out of 26—had a dominant effect on IHN. Given these observations, we believe our hypothesis to be correct. Expression of IHN symptoms is not a monogenic trait, but the combination of a moderate number of QTL with mostly dominant gene action is likely producing the skewed phenotypic distribution we have observed.

Given that most of the QTL were found in the susceptible parent, 'Atlantic', and were associated with decreasing IHN symptoms, the question remains; why is 'Atlantic' susceptible to IHN? There are at least two possibilities. First, though most 'Atlantic' QTL were associated with resistance, we did find markers associated with susceptibility, particularly those linked to the QTL on chromosome V and linkage group X; these regions may have enough influence on IHN to result in 'Atlantic' being susceptible. Second, it is also possible that these alleles associated with susceptibility interact with alleles at other loci which are not segregating in the B2721 population, thereby preventing their detection. In that case, it would be useful to develop a map in another population (such as NC206) to search for these additional loci. We also point out that 'Atlantic' has variable susceptibility to IHN; as mentioned earlier, Henninger et al. (2000) determined that 'Atlantic' is an unstable clone with respect to this trait. This phenomenon could have limited our ability to detect more QTL and/or QTL alleles associated with susceptibility. Future research on the genetics of IHN should perhaps utilize material that is more highly and stably susceptible. Certain members of the B2721 population fit these criteria, as does B2464-1, the susceptible parent of NC206.

Portability of QTL and markers

In order to test the utility of the markers in B2721 we screened several of them in a secondary population that also segregated for IHN. The QTL for IHN resistance on chromosome I was detected in the NC206 population, via the AFLP marker E38M58_135. A QTL allele for IHN susceptibility on chromosome V of B1829-5, represented by the SSR marker STI032_4, was also located in NC206. In this case, the SSR itself was not associated with IHN, but was closely linked in repulsion to an AFLP marker that was associated with susceptibility. In NC206, both of these markers were present in the susceptible parent. The fact that the parent was highly susceptible to IHN (more so than 'Atlantic') despite the presence of E38M58_135 suggests that the QTL on chromosome I does not have a major effect on IHN, or does so only in certain genetic backgrounds. Clearly, more markers from B2721 need to be tested in NC206 or other populations segregating for IHN, to determine how useful any markers derived from that population would be in selecting for IHN resistance in other genetic backgrounds. It is too early to tell whether or not IHN resistance/susceptibility is governed by a common or diverse set of loci, but these initial results indicate that at least some of the loci involved in IHN are common across these two populations.

Map characteristics

To date, the most comprehensive linkage maps for tetraploid potato are those developed by Bradshaw et al. (2008). They obtained map lengths of 1,202 cM for 12601ab1, the female parent, and 1,234 cM for 'Stirling', the male parent of the mapping population. Our estimates of 1,051.1 cM for 'Atlantic' and 969.5 cM for B1829-5 compare favorably with the estimates of Bradshaw et al. (2008). Khu et al. (2008) obtained longer distances for their tetraploid maps, 2940 and 1929 cM for the female and male parents, respectively. However, it is unclear whether these distances are based on the length of overall linkage groups, or the length of each homolog within a linkage group. The ultra high-density (UHD)

linkage maps of diploid potato assembled by van Os et al. (2006) were 751 and 773 cM for the female and male parents, respectively. Our map distances are slightly longer, in basic agreement with the observation by Bradshaw et al. (2008) that marker order between the UHD and their tetraploid maps was generally the same, but with longer map distances between markers.

Using a total of 14 SSR markers from tomato and potato, we were able to tag (in one or both parents) chromosomes I, II, III, IV, V, VI, VIII, IX, and XI. We were most successful in the case of chromosome III, where three SSR markers (SSR111, SSR22, and SSR300) were successfully mapped. Chromosomes VII, X, and XII were not tagged. Our maps contain large linkage groups, such as groups X and XII of 'Atlantic' and group VII in both parents (Figure 2), that likely correspond with the remaining three chromosomes. Using agarose gel electrophoresis, we putatively identified one SSR (two for chromosome VII) for each of these chromosomes, that was polymorphic in B2721. Marker STI008, located on chromosome VII, was unlinked in our study, and the second chromosome VII marker, STI064, was in actuality monomorphic. SSR4 and SSR20, located on chromosomes 10 and 12, respectively, did not amplify when using the M13-tailed primer method, a phenomenon that is rare, but known to occur (R. Bravo and J. Lyerly, personal communication). Cost constraints prohibited us from screening more SSRs, but we are confident that any future attempts to tag the three remaining chromosomes using B2721 would be successful. Khu et al. (2008), using a smaller population (92 individuals) and more SSR markers (95), were able to tag all 12 potato chromosomes.

Allelic effects

When a simple simplex or duplex dominant model does not adequately describe QTL effects, the six-mean models developed by TetraploidMap (representing the six possible ways a parent can transmit any two alleles to its offspring) can be difficult to interpret. Figure 3 illustrates the effects of the six genotypes for the *inc06* QTL on 'Atlantic'

chromosome V. Allele 3 appears to be epistatic to the other three alleles, while allele 4 seems to have a dominant negative effect over alleles 1 and 2. These estimates demonstrate the complex genetic effects present in polyploids, which are absent in diploids. QTL mapping software developed for diploid systems are designed to handle one 'homolog' at a time. This can also be done in polyploids but requires the presence of simplex markers on each homolog, in contrast to TetraploidMap, which incorporates all marker types and is therefore more efficient. If sufficient simplex markers were available, we would likely gain more information about intralocus interactions. Improved marker coverage may also minimize the phenomenon where a QTL is modeled with dominant gene action in one season, but with additive gene action in another (Table 3, linkage group X, sev06 vs. sev07) Although TetraploidMap is superior to other software in terms of its ability to incorporate dominant and co-dominant markers in an autotetraploid population, the IM model of TetraploidMap does not provide estimates of interactions between QTL. Again, the presence of more simplex markers covering all four homologs would allow for these more advanced analyses using software such as Qgene (Joehanes and Nelson 2008) or QTL Cartographer (Wang et al. 2007). Through the SOLCAP project (http://solcap.msu.edu), large numbers of sequence-specific, portable SNP markers will soon be available. These markers could be readily incorporated into the B2721 maps to improve map coverage and QTL analyses.

Maturity

There is a small, but significant correlation between maturity and internal heat necrosis, with earlier-maturing clones showing lower levels of IHN. We have detected a maturity QTL on chromosome V, in both parents. This chromosome also carries QTL for IHN incidence and severity, detected in both parents. The QTL for maturity were located near the distal end of the chromosome, with LOD peaks from 56 to 76 cM. The location of QTL for IHN severity/incidence are less precise, but range from 0 to 46 cM. We feel this is good evidence that the relationship between maturity and IHN, at least for these loci, is due

to loose linkage, and not pleiotropy. This does not rule out pleiotropic effects from other loci. For example, it is known that IHN symptoms get worse as the season progresses (Sterrett et al.1991b; P. McCord, personal observation). Individuals that complete their life cycle relatively early in the growing season may be able to escape some of the environmental stresses responsible for the disorder. Sterrett and Henninger (1997) discussed harvesting earlier in the season to avoid IHN symptoms, but pointed out that this tended to reduce yields. Early-maturing varieties that size quickly might be able to avoid this yield penalty. In any case, the fact remains that the correlation between IHN and maturity, though real, is relatively low in absolute terms; early maturity is not a cure for IHN.

Conclusion

Because of its significant economic impact, the difficulty of screening in the field, and its quantitative distribution, IHN is a good candidate for QTL analysis. The QTL mapping reported in this paper is an important first step towards understanding the genetics of the disorder, and developing markers for selection. As a prerequisite for QTL mapping, we successfully generated linkage maps of tetraploid potato, and anchored most of the linkage groups to the known *Solanum* chromosomes. We have reliably identified QTL for IHN incidence and/or severity on chromosomes IV and V, and linkage groups VII and X, and have shown that in most cases, these QTL exhibit dominant action for resistance, which likely explains the skewed phenotypic distribution of IHN. Markers linked to these QTL, and markers from other linkage groups identified using single-point ANOVA, are now candidates for further testing for their utility in selection. We have initiated this process by testing several of these markers in a secondary mapping population; in two cases, we have demonstrated that certain QTL are common to different breeding populations. Much research remains to be done to detect additional QTL and to better quantify the effects and possible interactions of the QTL we have already identified, and to test the efficacy of

markers linked to these QTL. The research we have presented provides a solid foundation for further pursuit of this important and intriguing disorder of potato.

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Table 1A. Correlations between IHN incidence and severity, and other agronomic traits measured in population B2721. P-values of correlations are listed in parentheses. Incidence = IHN incidence. Severity= IHN severity. SG = specific gravity. DM = dry matter. Texture = skin texture. Maturity = foliage maturity.

	Incidence	**Severity**	**Yield**	**SG**	**DM**	**Texture**	**Maturity**
Incidence		-0.95 (<0.0001)	0.13 (0.007)	-0.09 (0.05)	0.01 (0.81)	0.17 (0.0003)	-0.44 (<0.0001)
Severity	-0.95 (<0.0001)		-0.08 (0.09)	0.07 (0.13)	-0.03 (0.56)	-0.15 (0.002)	0.34 (<0.0001)

Table 1B. Correlations between IHN incidence and severity, and other agronomic traits measured in population NC206. P-values of correlations are listed in parentheses. Incidence = IHN incidence. Severity= IHN severity. SG = specific gravity. DM = dry matter. Texture = skin texture. Maturity = foliage maturity. Flesh = flesh color.

	Incidence	**Severity**	**Yield**	**SG**	**DM**	**Texture**	**Maturity**	**Flesh**
Incidence		-0.94 (<0.0001)	0.45 (<0.0001)	0.08 (0.15)	0.17 (0.002)	0.26 (<0.0001)	-0.36 (<0.0001)	-0.03 (0.56)
Severity	-0.94 (<0.0001)		-0.38 (<0.0001)	-0.08 (0.13)	-0.17 (0.003)	-0.21 (0.0002)	0.28 (<0.0001)	0.02 (0.72)

Table 2. List of AFLP primer combinations, with the number of markers generated by each combination. Primer codes are in Keygene format (see Figure 2 for URL). Not all markers in this table were used in map construction.

Combination	Markers
E32M32	6
E32M36	23
E32M37	11
E32M40	9
E32M41	15
E34M49	14
E34M52	14
E34M56	8
E36M32	11
E36M34	11
E36M52	16
E36M54	23
E36M56	4
E36M57	18
E36M60	7
E38M54	20

Combination	Markers
E38M55	3
E38M56	9
E38M58	18
E40M51	11
E40M52	10
E40M53	3
E40M59	18
E41M49	4
E41M50	32
E41M56	12
E41M57	11
E42M52	13
E42M53	7
E42M54	11
E42M55	20
E42M60	12

Combination	Markers
E44M52	17
E44M54	2
E44M56	8
E45M48	25
E45M52	27
E45M53	21
E45M58	25
E45M60	7
E45M61	9
P12M32	21
P12M36	14
P12M37	11
P12M39	12
P12M42	14
P12M43	14
P12M44	17

Table 3. List of QTL detected for IHN severity, IHN incidence, and foliage maturity. Abbreviations: sev06, 07, 08= IHN severity 2006, 2007, 2008; inc06, 07, 08= IHN incidence 2006, 2007, 2008; mat06, 07, 08= foliage maturity 2006, 2007, 2008; LG = linkage group/chromosome; NE = not estimable (occurs when a secondary LOD peak is detected); SE = standard error. LOD scores with an asterisk were between the 90 and 95th percentile in a permutation test of 100 iterations. Scores with the ^ symbol had a LOD score of at least 1.8, and were reported if a QTL was found in a similar location at least one other year, and met the more stringent LOD score and permutation test criteria. The standard errors of trait means are in parentheses. For QTL with additive effects, there are six means, one for each QTL genotype (see Figure 3); they are not listed in this table.

Trait	Parent	LG	Position (cM)	LOD score	R^2	Dosage/ effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
inc06	Atlantic	V	32	4.57	13.1	Additive		
inc06	Atlantic	V	18	4.13	NE	NE		
inc06	Atlantic	X	74	2.55*	9.1	Duplex, dominant	10.6 (1.8)	30.4 (5.3)
inc06	B1829-5	VII	20	2.6*	7.3	Duplex, dominant	10.3 (1.6)	26.3 (4.2)
inc07	Atlantic	IV	10	2.33*	14.1	Duplex, dominant	14.9 (2.0)	42.5 (5.0)
inc07	Atlantic	V	38	4.26	14.5	Additive		
inc07	Atlantic	X	74	2.22^	4.9	Additive		
inc07	B1829-5	V	8	3.49	8.6	Additive		
inc07	B1829-5	V	24	3.2*	NE	NE		
inc07	B1829-5	VII	38	1.89^	6.1	Duplex, dominant	15.8 (2.2)	33.4 (5.0)

Table 3- continued

Trait	Parent	LG	Position (cM)	LOD score	R^2	Dosage/ effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
inc08	Atlantic	IV	10	7.63	32.5	Duplex, dominant	3.3 (0.9)	25.9 (2.4)
inc08	Atlantic	X	74	1.90^	6.8	Duplex, dominant	4.2 (1.2)	15.3 (3.5)
inc08	B1829-5	VII	38	2.45*	7.7	Duplex, dominant	4.4 (1.1)	14.3 (2.5)
sev06	Atlantic	IV	18	2.41*	7.6	Duplex, dominant	8.77 (0.05)	8.23 (0.14)
sev06	Atlantic	V	18	4.06	9.3	Additive		
sev06	Atlantic	X	74	2.90*	10.4	Duplex, dominant	8.76 (0.06)	8.03 (0.18)
sev06	B1829-5	VII	20	4.71	13.3	Duplex, dominant	8.79 (0.05)	8.05 (0.14)
sev06	B1829-5	VII	34	3.53	NE	NE		
sev07	Atlantic	IV	10	4	21.3	Duplex, dominant	8.64 (0.07)	7.34 (0.19)
sev07	Atlantic	V	46	3.27	17.2	Duplex, dominant	8.62 (0.08)	7.34 (0.22)
sev07	Atlantic	VII	84	5.76	29.4	Duplex, dominant	8.65 (0.08)	7.07 (0.20)
sev07	B1829-5	V	0	3.22*	6.7	Additive		

Table 3- continued

Trait	Parent	LG	Position (cM)	LOD score	R^2	Dosage/effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
sev07	B1829-5	VII	36	2.22^	8.5	Duplex, dominant	8.60 (0.08)	7.83 (0.18)
sev08	Atlantic	IV	10	8.56	35.2	Duplex, dominant	8.94 (0.02)	8.33 (0.06)
sev08	Atlantic	V	38	2.93*	7.5	Additive		
sev08	B1829-5	VII	38	3.35*	7.7	Additive		
mat07	Atlantic	II	38	1.91^	8.0	Simplex, dominant	66.3 (2.9)	78.7 (2.1)
mat07	Atlantic	III	90	4.13	10.4	Additive		
mat07	Atlantic	V	76	4.2	14.3	Duplex, dominant	71.6 (1.7)	90.7 (3.5)
mat07	Atlantic	XII	86	1.84^	5.3	Duplex, dominant	72.9 (1.8)	85.8 (3.9)
mat07	B1829-5	V	56	3.23	9.6	Duplex, dominant	77.6 (1.7)	61.5 (3.5)
mat08	Atlantic	II	36	2.33*	11.7	Simplex, dominant	66.9 (4.1)	88.8 (3.1)
mat08	Atlantic	III	6	4.5	13.8	Simplex, dominant	94.0 (3.4)	71.4 (3.3)
mat08	Atlantic	V	74	3.83	13.1	Duplex, dominant	76.0 (2.6)	103.8 (5.3)
mat08	B1829-5	V	56	5.23	15.6	Duplex, dominant	87.1 (2.5)	57.0 (5.0)

Table 4. List of single markers declared significant for IHN severity/incidence, and foliage maturity. Markers were tested using single-point ANOVA, and were declared significant if they had a p-value <0.01 in a single year, or <0.05 in multiple years. Mean(0) = mean of individuals with marker genotype 0 (absent). Mean(1) = mean of individuals with marker genotype 1 (present). SED = standard error of the difference between marker class means.

Trait	Marker	Parent	LG	p-value	Mean(0)	Mean(1)	SED
inc06	E45M61_229.2	B1829-5	I	0.009	17.7	8.6	3.0
inc06	E41M50_162.3	B1829-5	I	0.017	16.9	8.5	3.0
inc06	P12M36_619.1	B1829-5	XII	0.008	17.1	8.9	3.0
inc07	E41M50_162.3	B1829-5	I	0.002	25.9	13.6	4.0
inc07	E38M58_135	B1829-5	I	0.031	24.7	15.5	4.2
inc07	E36M32_203.5	B1829-5	I	0.015	24.5	14.6	4.0
inc07	E34M52_160.6	B1829-5	XI	0.025	14.0	23.8	4.3
inc07	SSR46	B1829-5	XI	0.01	12.1	22.6	4.0
inc08	E38M58_135.0	B1829-5	I	0.027	8.9	4.2	2.1
inc08	E41M50_162.3	B1829-5	I	0.007	9.2	3.7	2.0
inc08	E34M52_160.6	B1829-5	XI	0.011	3.1	8.8	2.2
inc08	SSR46	B1829-5	XI	0.004	2.2	8.1	2.0
sev06	E41M50_162.3	B1829-5	I	0.017	8.56	8.81	0.10
sev06	E45M61_229.2	B1829-5	I	0.009	8.53	8.81	0.11
sev06	E36M32_203.5	B1829-5	I	0.049	8.59	8.77	0.11

Table 4- continued

Trait	Marker	Parent	LG	p-value	Mean(0)	Mean(1)	SED
sev07	E41M50_162.3	B1829-5	I	0.002	8.20	8.67	0.15
sev07	E36M32_203.5	B1829-5	I	0.004	8.22	8.66	0.15
sev07	P12M42_104.0	B1829-5	X	0.004	8.69	8.40	0.17
sev08	E41M50_162.3	B1829-5	I	0.006	8.78	8.93	0.05
sev08	E38M58_135.0	B1829-5	I	0.027	8.79	8.91	0.05
sev08	E34M52_160.6	B1829-5	XI	0.034	8.93	8.81	0.06
sev08	SSR46	B1829-5	XI	0.009	8.95	8.82	0.05
mat07	E45M58_101.7	Atlantic	IX	0.008	81.8	72.0	3.6
mat07	E38M54_235.4	B1829-5	I	0.032	81.8	72.8	4.2
mat07	E38M58_100.0C	B1829-5	X	0.015	82.4	72.5	4.0
mat07	E45M60_81.3	B1829-5	XII	0.021	81.6	72.6	3.9
mat08	E38M54_235.4	B1829-5	I	0.004	96.2	78.1	6.1
mat08	E38M58_100.0C	B1829-5	X	0.020	92.6	78.5	6.0
mat08	E45M60_81.3	B1829-5	XII	0.041	89.9	77.9	5.8

Table 5. List of single markers tested in NC206, and found to be significantly associated with IHN incidence and severity.

Trait	Marker	Parent	p-value	Mean(0)	Mean(1)	SED
inc08	E38M58_135	B2464-1	0.004	5.8	1.7	1.4
sev07	E38M58_135	B2464-1	0.017	8.39	8.68	0.12
sev08	E38M58_135	B2464-1	0.006	8.88	8.97	0.03

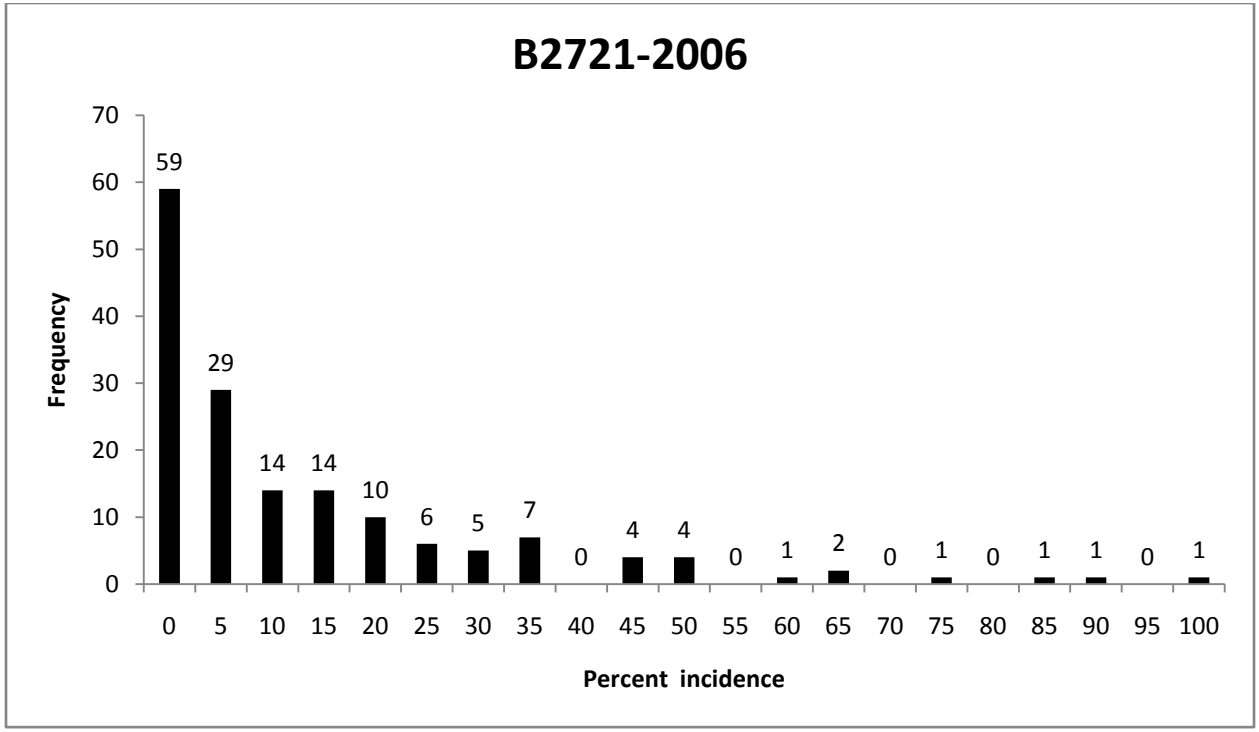


Figure 1A. Frequency distribution for IHN incidence in B2721, 2006 season.

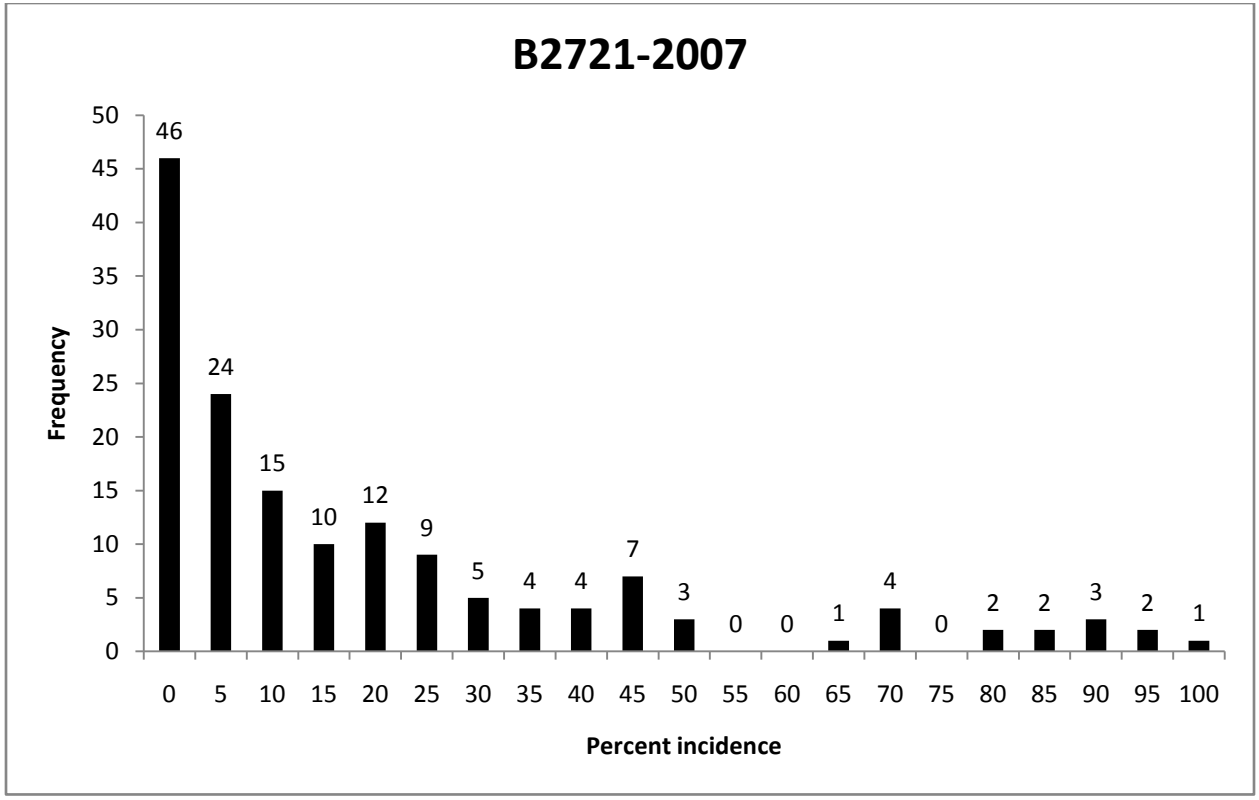


Figure 1B. Frequency distribution for IHN incidence in B2721, 2007 season.

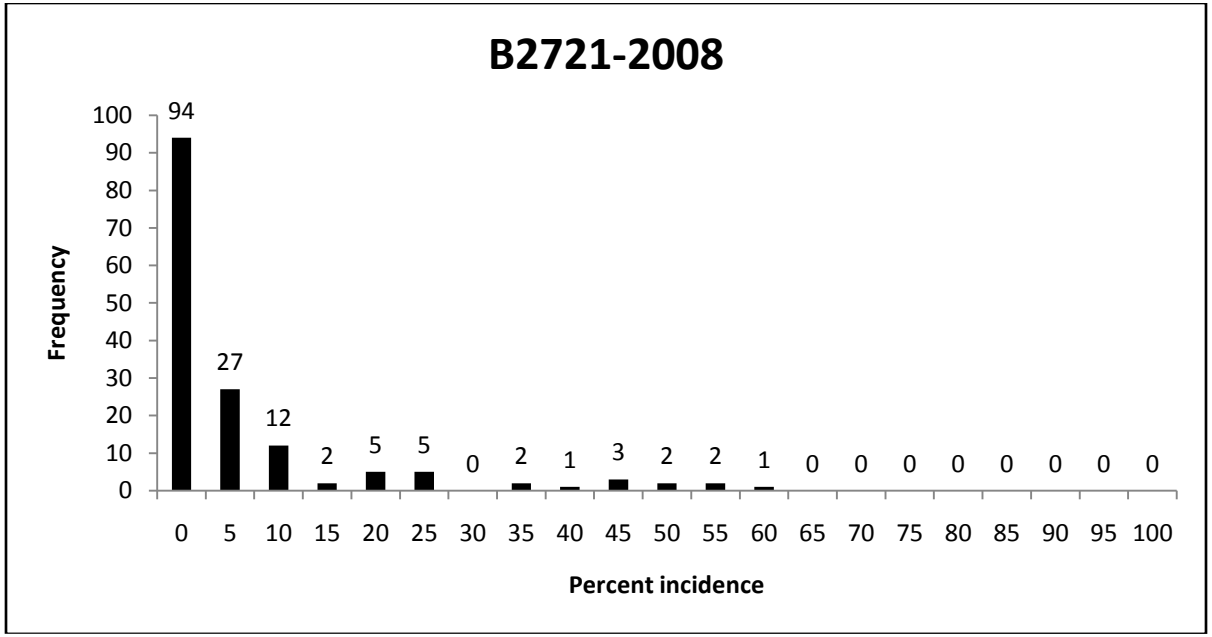


Figure 1C. Frequency distribution for IHN incidence in B2721, 2008 season.

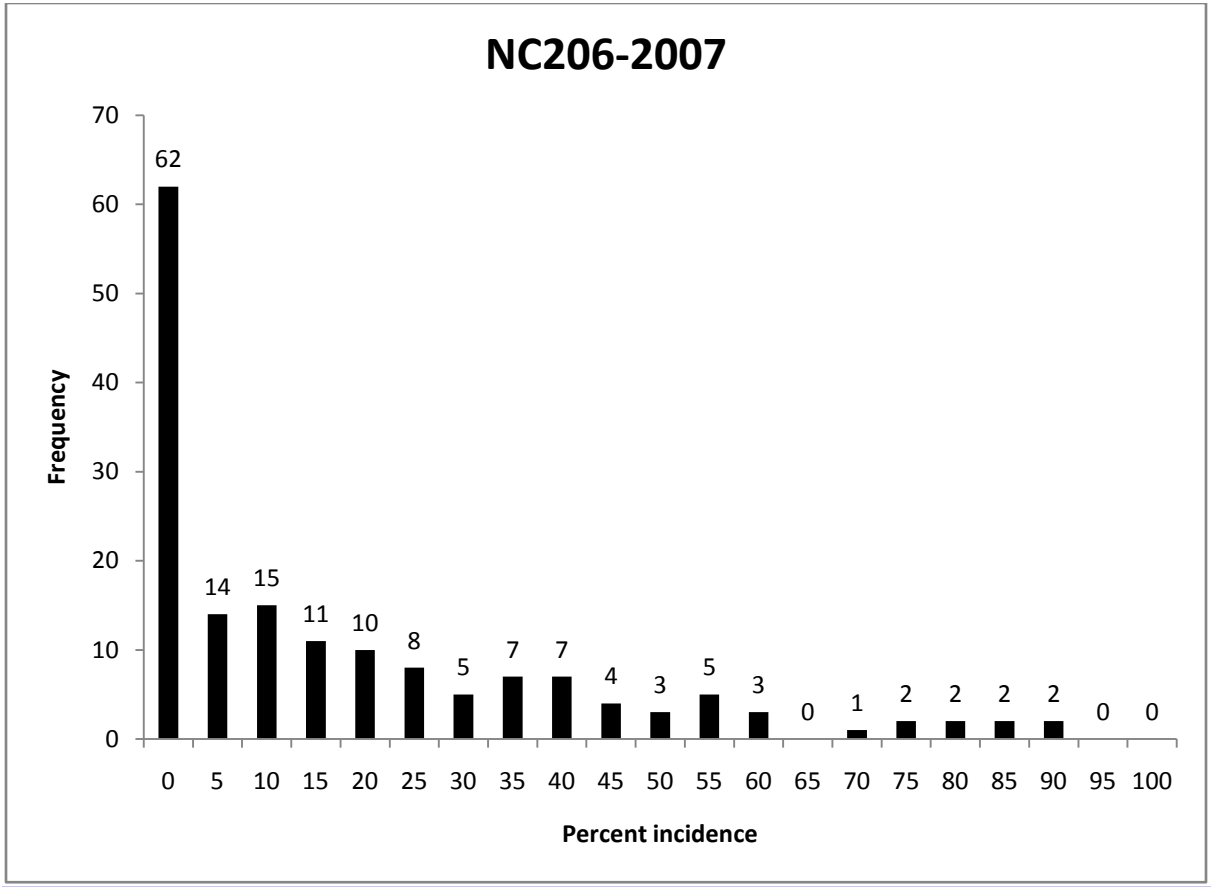


Figure 1D. Frequency distribution for IHN incidence in NC206, 2007 season.

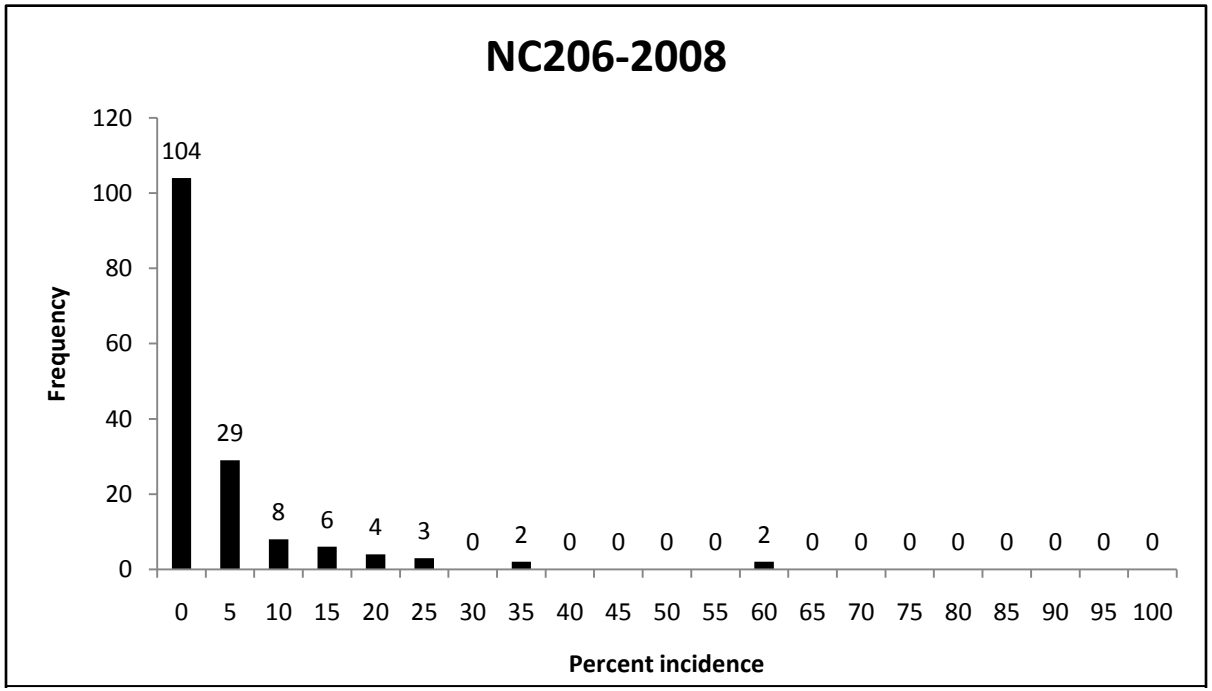
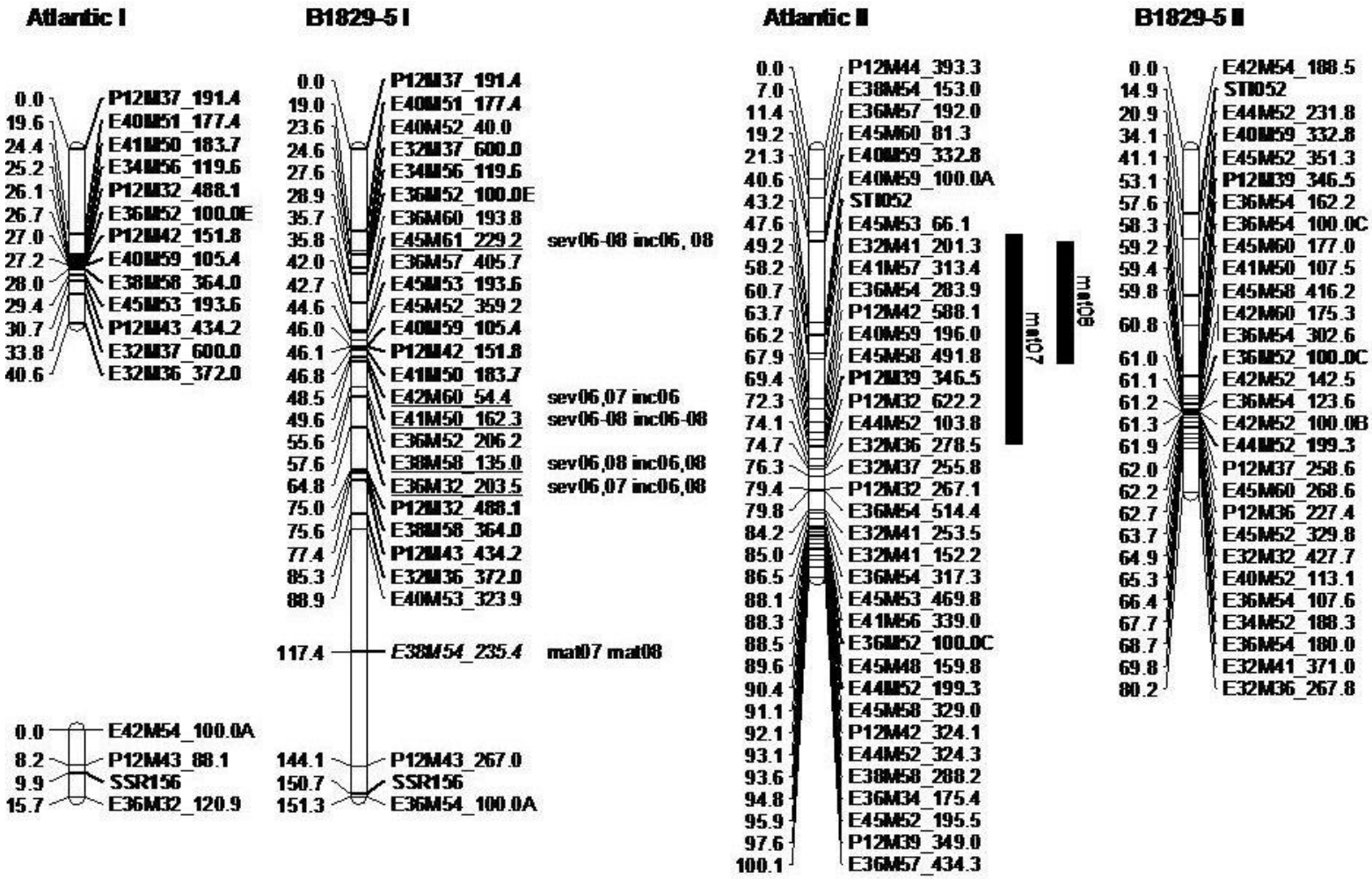
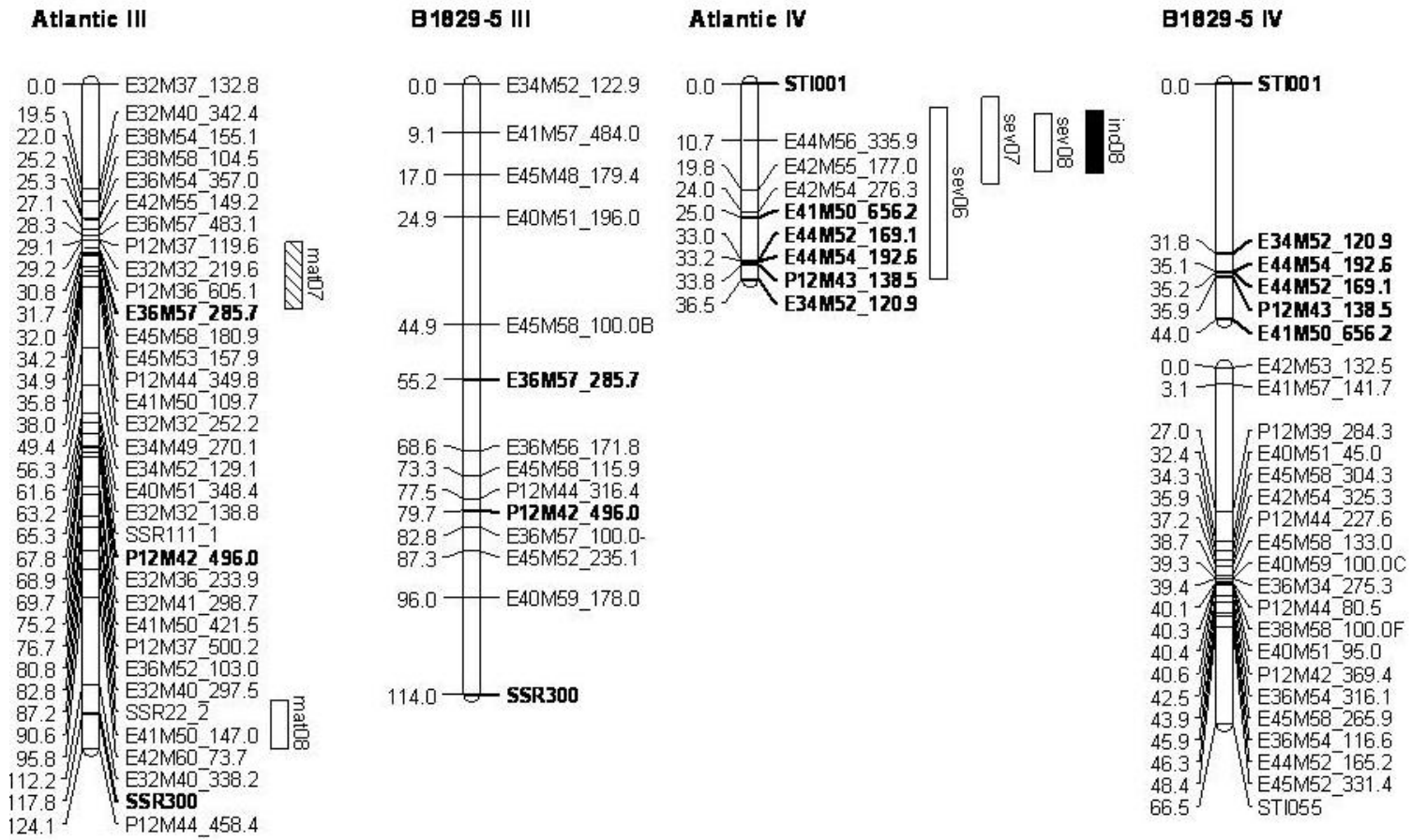


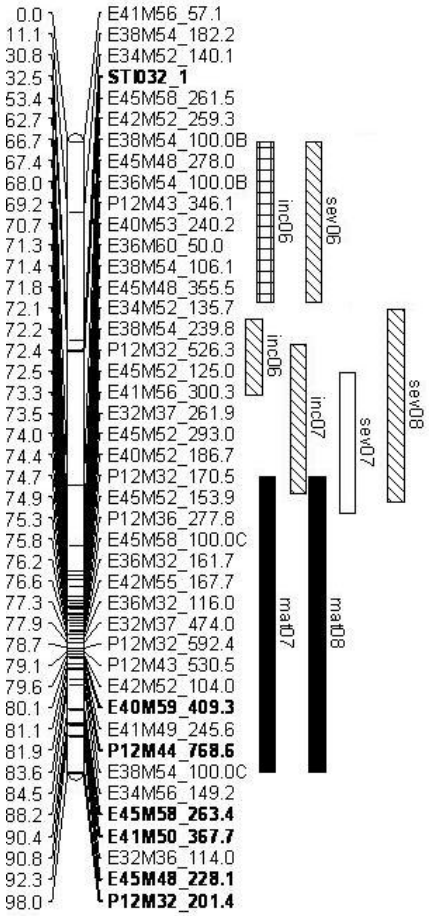
Figure 1E. Frequency distribution for IHN incidence in NC206, 2008 season.

Figure 2. Linkage maps of 'Atlantic' and B1829-5. Unless specified by the term 'group', Roman numerals refer to actual chromosomes. AFLP markers are denoted by the Keygene system of primer combinations (www.keygene.com/keygene/pdf/KF%20Primer%20enzyme%20combinations.pdf). The number after the underscore symbol is the approximate size in base pairs. Size estimates followed by letters refer to markers that were shorter than the shortest size marker, and are ranked alphabetically by size (A = largest, then B, and so forth). Tomato SSR markers are denoted by the prefix 'SSR', and potato SSR markers by the prefix 'STI'. Markers in bold type indicate SSR or double-simplex AFLP markers segregating in both parents, which were used to align the parental maps. Significant single markers are annotated by the trait(s) they are associated with, and are underlined if they affect the trait in a positive direction, or italicized if they have a negative effect. QTL are identified by shaded bars which represent a 1-LOD interval. Bar shading codes are the following: Black = dominant negative effect; white = dominant positive effect; diagonal lines = complex additive effect; horizontal lines = non-estimable effect. Maps were drawn using MapChart (Voorrips 2002), with minor adjustments made using GIMP (www.gimp.org).

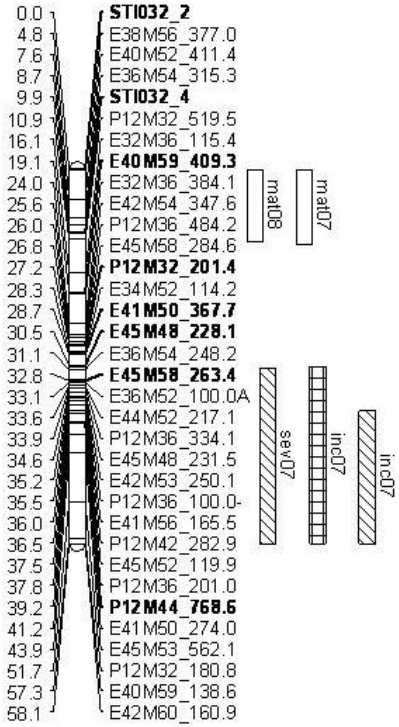

Atlantic I
0.0 P12M37_191.4
19.6 E40M51_177.4
24.4 E41M50_183.7
25.2 E34M56_119.6
26.1 P12M32_488.1
26.7 E36M52_100.0E
27.0 P12M42_151.8
27.2 E40M59_105.4
28.0 E38M58_364.0
29.4 E45M53_193.6
30.7 P12M43_434.2
33.8 E32M37_600.0
40.6 E32M36_372.0
0.0 E42M54_100.0A
8.2 P12M43_88.1
9.9 SSR156
15.7 E36M32_120.9
B1829-5 I
0.0 P12M37_191.4
19.0 E40M51_177.4
23.6 E40M52_40.0
24.6 E32M37_600.0
27.6 E34M56_119.6
28.9 E36M52_100.0E
35.7 E36M60_193.8
35.8 E45M61_229.2 sev06-08 inc06, 08
42.0 E36M57_405.7
42.7 E45M53_193.6
44.6 E45M52_359.2
46.0 E40M59_105.4
46.1 P12M42_151.8
46.8 E41M50_183.7
48.5 E42M60_54.4 sev06,07 inc06
49.6 E41M50_162.3 sev06-08 inc06-08
55.6 E36M52_206.2
57.6 E38M58_135.0 sev06,08 inc06,08
64.8 E36M32_203.5 sev06,07 inc06,08
75.0 P12M32_488.1
75.6 E38M58_364.0
77.4 P12M43_434.2
85.3 E32M36_372.0
88.9 E40M53_323.9
117.4 E38M54_235.4 mat07 mat08
144.1 P12M43_267.0
150.7 SSR156
151.3 E36M54_100.0A
Atlantic II
0.0 P12M44_393.3
7.0 E38M54_153.0
11.4 E36M57_192.0
19.2 E45M60_81.3
21.3 E40M59_332.8
40.6 E40M59_100.0A
43.2 STI052
47.6 E45M53_66.1
49.2 E32M41_201.3
58.2 E41M57_313.4
60.7 E36M54_283.9
63.7 P12M42_588.1
66.2 E40M59_196.0
67.9 E45M58_491.8
69.4 P12M39_346.5
72.3 P12M32_622.2
74.1 E44M52_103.8
74.7 E32M36_278.5
76.3 E32M37_255.8
79.4 P12M32_267.1
79.8 E36M54_514.4
84.2 E32M41_253.5
85.0 E32M41_152.2
86.5 E36M54_317.3
88.1 E45M53_469.8
88.3 E41M56_339.0
88.5 E36M52_100.0C
89.6 E45M48_159.8
90.4 E44M52_199.3
91.1 E45M58_329.0
92.1 P12M42_324.1
93.1 E44M52_324.3
93.6 E38M58_288.2
94.8 E36M34_175.4
95.9 E45M52_195.5
97.6 P12M39_349.0
100.1 E36M57_434.3
mat07
mat08
B1829-5 II
0.0 E42M54_188.5
14.9 STI052
20.9 E44M52_231.8
34.1 E40M59_332.8
41.1 E45M52_351.3
53.1 P12M39_346.5
57.6 E36M54_162.2
58.3 E36M54_100.0C
59.2 E45M60_177.0
59.4 E41M50_107.5
59.8 E45M58_416.2
60.8 E42M60_175.3
E36M54_302.6
61.0 E36M52_100.0C
61.1 E42M52_142.5
61.2 E36M54_123.6
61.3 E42M52_100.0B
61.9 E44M52_199.3
62.0 P12M37_258.6
62.2 E45M60_268.6
62.7 P12M36_227.4
63.7 E45M52_329.8
64.9 E32M32_427.7
65.3 E40M52_113.1
66.4 E36M54_107.6
67.7 E34M52_188.3
68.7 E36M54_180.0
69.8 E32M41_371.0
80.2 E32M36_267.8


Atlantic III
0.0 E32M37_132.8
19.5 E32M40_342.4
22.0 E38M54_155.1
25.2 E38M58_104.5
25.3 E36M54_357.0
27.1 E42M55_149.2
28.3 E36M57_483.1
29.1 P12M37_119.6
29.2 E32M32_219.6
30.8 P12M36_605.1
31.7 E36M57_285.7
32.0 E45M58_180.9
34.2 E45M53_157.9
34.9 P12M44_349.8
35.8 E41M50_109.7
38.0 E32M32_252.2
49.4 E34M49_270.1
56.3 E34M52_129.1
61.6 E40M51_348.4
63.2 E32M32_138.8
65.3 SSR111_1
67.8 P12M42_496.0
68.9 E32M36_233.9
69.7 E32M41_298.7
75.2 E41M50_421.5
76.7 P12M37_500.2
80.8 E36M52_103.0
82.8 E32M40_297.5
87.2 SSR22_2
90.6 E41M50_147.0
95.8 E42M60_73.7
112.2 E32M40_338.2
117.8 SSR300
124.1 P12M44_458.4
mat07
mat08
B1829-5 III
0.0 E34M52_122.9
9.1 E41M57_484.0
17.0 E45M48_179.4
24.9 E40M51_196.0
44.9 E45M58_100.0B
55.2 E36M57_285.7
68.6 E36M56_171.8
73.3 E45M58_115.9
77.5 P12M44_316.4
79.7 P12M42_496.0
82.8 E36M57_100.0-
87.3 E45M52_235.1
96.0 E40M59_178.0
114.0 SSR300
Atlantic IV
0.0 STI001
10.7 E44M56_335.9
19.8 E42M55_177.0
24.0 E42M54_276.3
25.0 E41M50_656.2
33.0 E44M52_169.1
33.2 E44M54_192.6
33.8 P12M43_138.5
36.5 E34M52_120.9
sew06
sew07
sew08
ind08
B1829-5 IV
0.0 STI001
31.8 E34M52_120.9
35.1 E44M54_192.6
35.2 E44M52_169.1
35.9 P12M43_138.5
44.0 E41M50_656.2
0.0 E42M53_132.5
3.1 E41M57_141.7
27.0 P12M39_284.3
32.4 E40M51_45.0
34.3 E45M58_304.3
35.9 E42M54_325.3
37.2 P12M44_227.6
38.7 E45M58_133.0
39.3 E40M59_100.0C
39.4 E36M34_275.3
40.1 P12M44_80.5
40.3 E38M58_100.0F
40.4 E40M51_95.0
40.6 P12M42_369.4
42.5 E36M54_316.1
43.9 E45M58_265.9
45.9 E36M54_116.6
46.3 E44M52_165.2
48.4 E45M52_331.4
66.5 STI055

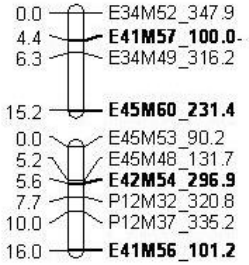
Atlantic V



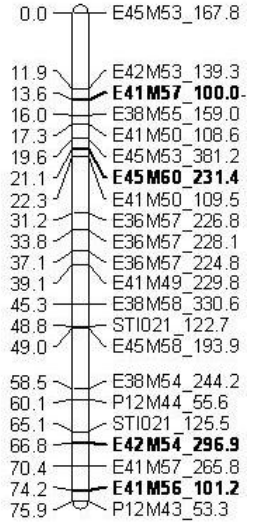
B1829-5 V

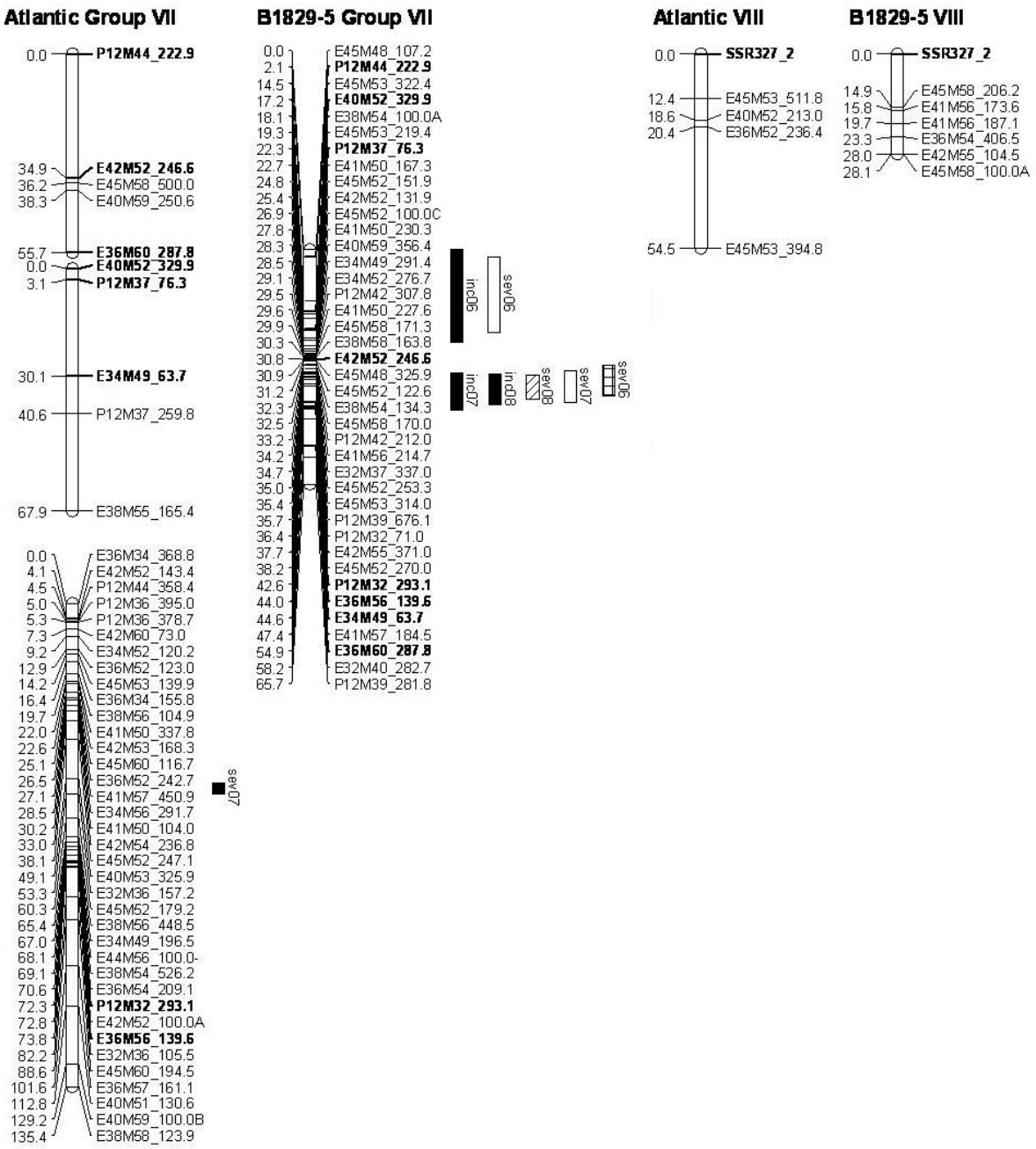


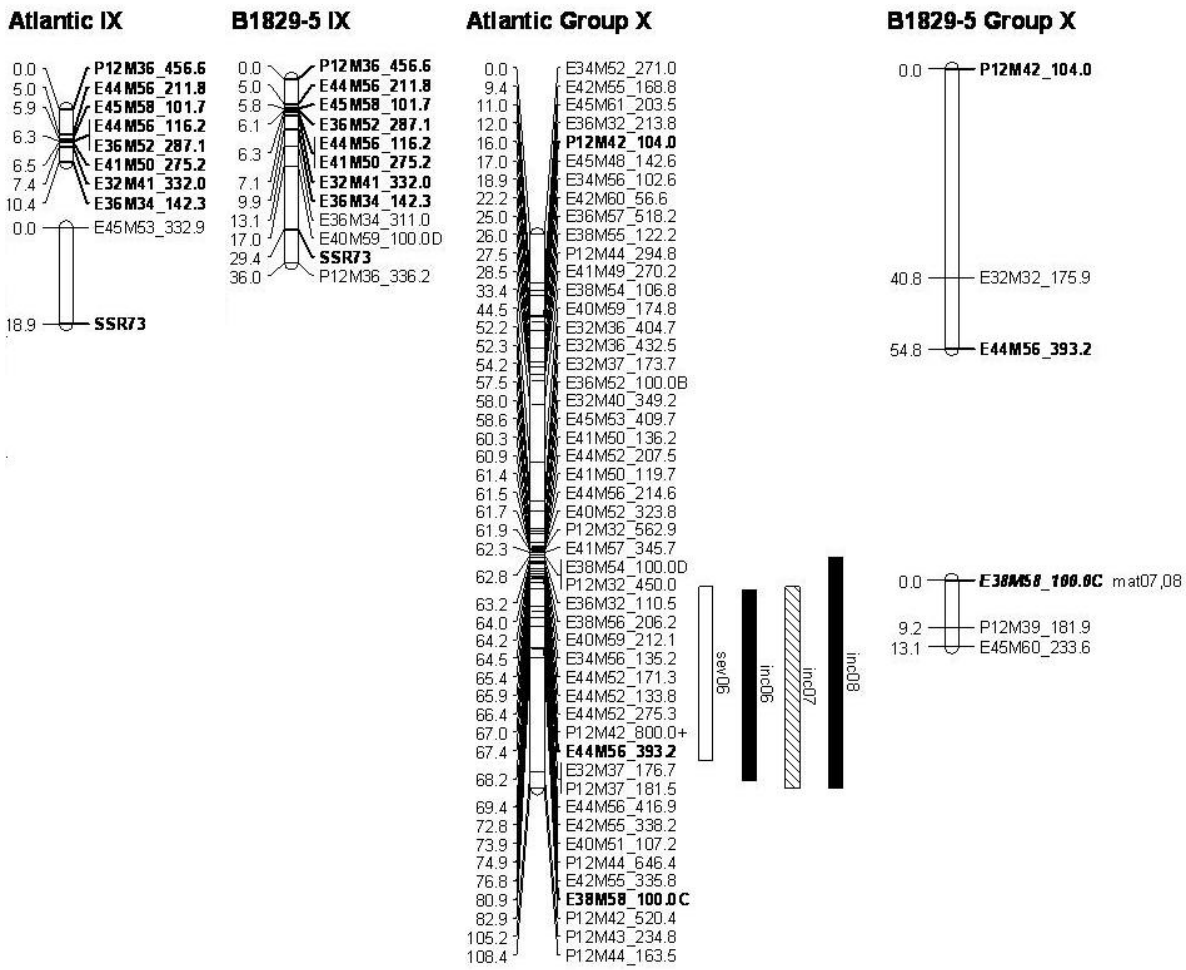
Atlantic VI

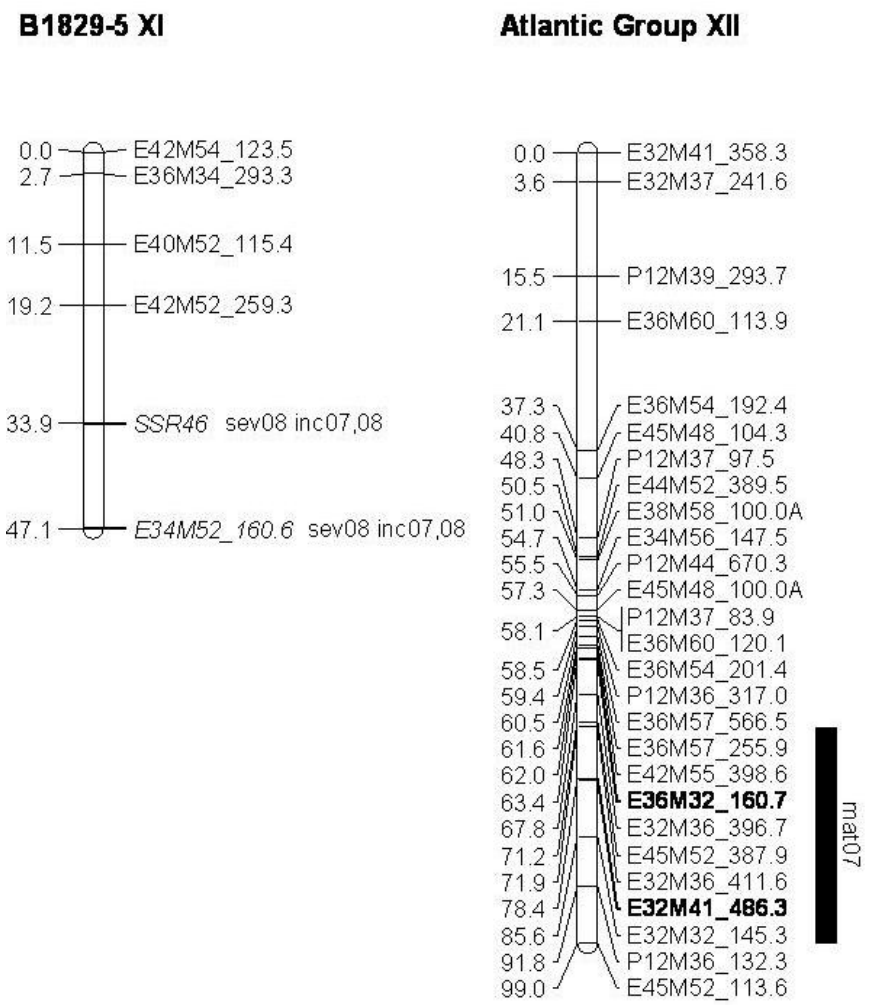


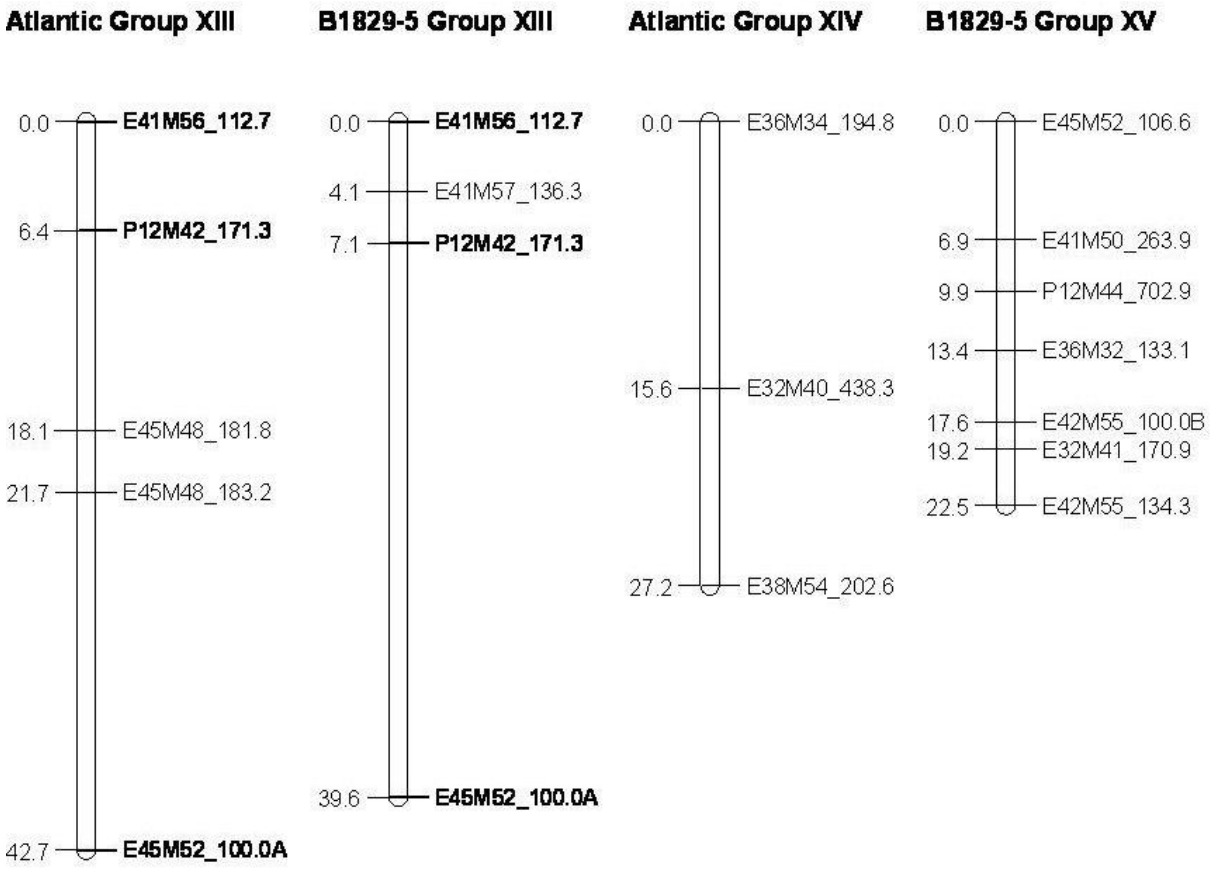
B1829-5 VI




Atlantic Group VII
0.0 P12M44_222.9
34.9 E42M52_246.6
36.2 E45M58_500.0
38.3 E40M59_250.6
55.7 E36M60_287.8
0.0 E40M52_329.9
3.1 P12M37_76.3
30.1 E34M49_63.7
40.6 P12M37_259.8
67.9 E38M55_165.4
0.0 E36M34_368.8
4.1 E42M52_143.4
4.5 P12M44_358.4
5.0 P12M36_395.0
5.3 P12M36_378.7
7.3 E42M60_73.0
9.2 E34M52_120.2
12.9 E36M52_123.0
14.2 E45M53_139.9
16.4 E36M34_155.8
19.7 E38M56_104.9
22.0 E41M50_337.8
22.6 E42M53_168.3
25.1 E45M60_116.7
26.5 E36M52_242.7
27.1 E41M57_450.9
28.5 E34M56_291.7
30.2 E41M50_104.0
33.0 E42M54_236.8
38.1 E45M52_247.1
49.1 E40M53_325.9
53.3 E32M36_157.2
60.3 E45M52_179.2
65.4 E38M56_448.5
67.0 E34M49_196.5
68.1 E44M56_100.0-
69.1 E38M54_526.2
70.6 E36M54_209.1
72.3 P12M32_293.1
72.8 E42M52_100.0A
73.8 E36M56_139.6
82.2 E32M36_105.5
88.6 E45M60_194.5
101.6 E36M57_161.1
112.8 E40M51_130.6
129.2 E40M59_100.0B
135.4 E38M58_123.9
sev07
B1829-5 Group VII
0.0 E45M48_107.2
2.1 P12M44_222.9
14.5 E45M53_322.4
17.2 E40M52_329.9
18.1 E38M54_100.0A
19.3 E45M53_219.4
22.3 P12M37_76.3
22.7 E41M50_167.3
24.8 E45M52_151.9
25.4 E42M52_131.9
26.9 E45M52_100.0C
27.8 E41M50_230.3
28.3 E40M59_356.4
28.5 E34M49_291.4
29.1 E34M52_276.7
29.5 P12M42_307.8
29.6 E41M50_227.6
29.9 E45M58_171.3
30.3 E38M58_163.8
30.8 E42M52_246.6
30.9 E45M48_325.9
31.2 E45M52_122.6
32.3 E38M54_134.3
32.5 E45M58_170.0
33.2 P12M42_212.0
34.2 E41M56_214.7
34.7 E32M37_337.0
35.0 E45M52_253.3
35.4 E45M53_314.0
35.7 P12M39_676.1
36.4 P12M32_71.0
37.7 E42M55_371.0
38.2 E45M52_270.0
42.6 P12M32_293.1
44.0 E36M56_139.6
44.6 E34M49_63.7
47.4 E41M57_184.5
54.9 E36M60_287.8
58.2 E32M40_282.7
65.7 P12M39_281.8
ind06
sev06
ind07
ind08
sev08
sev07
sev06
Atlantic VIII
0.0 SSR327_2
12.4 E45M53_511.8
18.6 E40M52_213.0
20.4 E36M52_236.4
54.5 E45M53_394.8
B1829-5 VIII
0.0 SSR327_2
14.9 E45M58_206.2
15.8 E41M56_173.6
19.7 E41M56_187.1
23.3 E36M54_406.5
28.0 E42M55_104.5
28.1 E45M58_100.0A


Atlantic IX
0.0 P12M36_456.6
5.0 E44M56_211.8
5.9 E45M58_101.7
E44M56_116.2
6.3 E36M52_287.1
6.5 E41M50_275.2
7.4 E32M41_332.0
10.4 E36M34_142.3
0.0 E45M53_332.9
18.9 SSR73
B1829-5 IX
0.0 P12M36_456.6
5.0 E44M56_211.8
5.8 E45M58_101.7
6.1 E36M52_287.1
6.3 E44M56_116.2
E41M50_275.2
7.1 E32M41_332.0
9.9 E36M34_142.3
13.1 E36M34_311.0
17.0 E40M59_100.0D
29.4 SSR73
36.0 P12M36_336.2
Atlantic Group X
0.0 E34M52_271.0
9.4 E42M55_168.8
11.0 E45M61_203.5
12.0 E36M32_213.8
16.0 P12M42_104.0
17.0 E45M48_142.6
18.9 E34M56_102.6
22.2 E42M60_56.6
25.0 E36M57_518.2
26.0 E38M55_122.2
27.5 P12M44_294.8
28.5 E41M49_270.2
33.4 E38M54_106.8
44.5 E40M59_174.8
52.2 E32M36_404.7
52.3 E32M36_432.5
54.2 E32M37_173.7
57.5 E36M52_100.0B
58.0 E32M40_349.2
58.6 E45M53_409.7
60.3 E41M50_136.2
60.9 E44M52_207.5
61.4 E41M50_119.7
61.5 E44M56_214.6
61.7 E40M52_323.8
61.9 P12M32_562.9
62.3 E41M57_345.7
E38M54_100.0D
62.8 P12M32_450.0
63.2 E36M32_110.5
64.0 E38M56_206.2
64.2 E40M59_212.1
64.5 E34M56_135.2
65.4 E44M52_171.3
65.9 E44M52_133.8
66.4 E44M52_275.3
67.0 P12M42_800.0+
67.4 E44M56_393.2
68.2 E32M37_176.7
P12M37_181.5
69.4 E44M56_416.9
72.8 E42M55_338.2
73.9 E40M51_107.2
74.9 P12M44_646.4
76.8 E42M55_335.8
80.9 E38M58_100.0C
82.9 P12M42_520.4
105.2 P12M43_234.8
108.4 P12M44_163.5
swv06
inc06
inc07
inc08
B1829-5 Group X
0.0 P12M42_104.0
40.8 E32M32_175.9
54.8 E44M56_393.2
0.0 E38M58_100.0C mat07,08
9.2 P12M39_181.9
13.1 E45M60_233.6


B1829-5 XI
0.0 E42M54_123.5
2.7 E36M34_293.3
11.5 E40M52_115.4
19.2 E42M52_259.3
33.9 SSR46 sev08 inc07,08
47.1 E34M52_160.6 sev08 inc07,08
Atlantic Group XII
0.0 E32M41_358.3
3.6 E32M37_241.6
15.5 P12M39_293.7
21.1 E36M60_113.9
37.3 E36M54_192.4
40.8 E45M48_104.3
48.3 P12M37_97.5
50.5 E44M52_389.5
51.0 E38M58_100.0A
54.7 E34M56_147.5
55.5 P12M44_670.3
57.3 E45M48_100.0A
58.1 P12M37_83.9
E36M60_120.1
58.5 E36M54_201.4
59.4 P12M36_317.0
60.5 E36M57_566.5
61.6 E36M57_255.9
62.0 E42M55_398.6
63.4 E36M32_160.7
67.8 E32M36_396.7
71.2 E45M52_387.9
71.9 E32M36_411.6
78.4 E32M41_486.3
85.6 E32M32_145.3
91.8 P12M36_132.3
99.0 E45M52_113.6
mat07
B1829-5 Group XII
0.0 E45M53_360.4
15.8 E45M60_81.3 mat07,08
17.8 E36M57_393.8
19.0 E42M52_307.8
22.1 E38M58_100.0E
23.0 E38M58_155.9
23.6 E44M52_332.1
24.2 E45M53_197.1
24.6 E38M54_258.5
25.1 E38M56_173.3
25.4 P12M37_565.2
25.7 E36M32_217.3
26.6 E45M48_240.1
27.6 E40M59_100.0E
28.3 E45M53_95.0
30.2 E32M41_238.1
30.6 P12M36_619.1 inc06
35.6 E36M32_160.7
38.5 E32M41_486.3
64.7 E42M60_75.3


Atlantic Group XIII
0.0 E41M56_112.7
6.4 P12M42_171.3
18.1 E45M48_181.8
21.7 E45M48_183.2
42.7 E45M52_100.0A
B1829-5 Group XIII
0.0 E41M56_112.7
4.1 E41M57_136.3
7.1 P12M42_171.3
39.6 E45M52_100.0A
Atlantic Group XIV
0.0 E36M34_194.8
15.6 E32M40_438.3
27.2 E38M54_202.6
B1829-5 Group XV
0.0 E45M52_106.6
6.9 E41M50_263.9
9.9 P12M44_702.9
13.4 E36M32_133.1
17.6 E42M55_100.0B
19.2 E32M41_170.9
22.5 E42M55_134.3

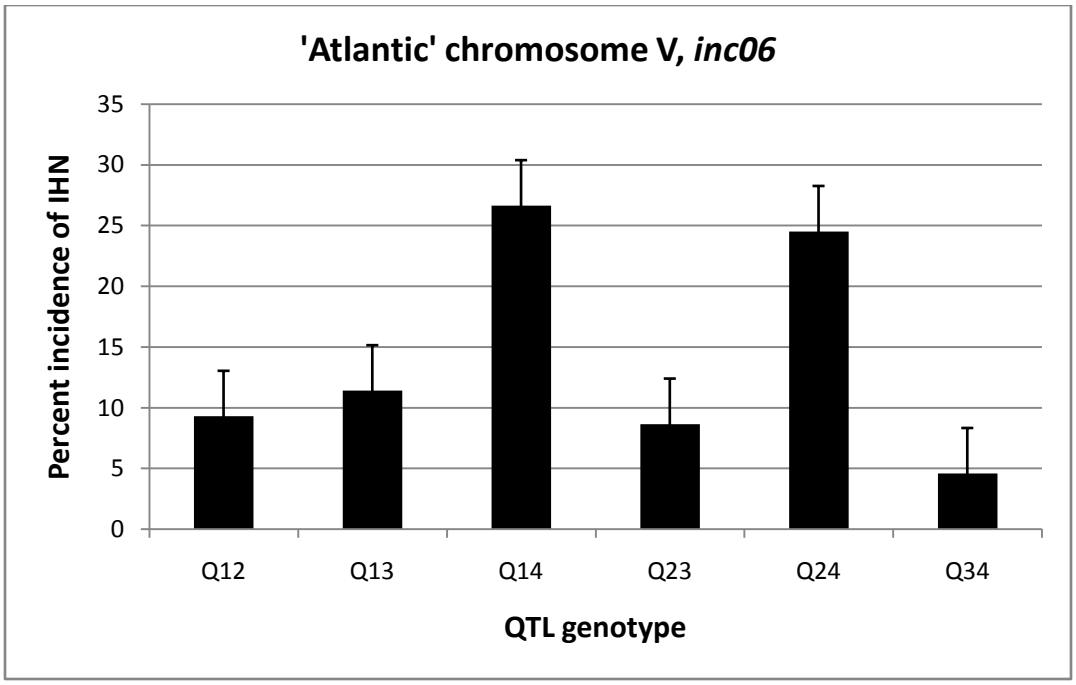


Figure 3. The effects of six possible QTL genotypes on the incidence of IHN in 2006. This QTL is centered at 32cM on chromosome V. Error bars represent an average of the standard errors of all genotypes. 'Q' refers to the putative QTL; 1-4 refer to the four homologs of each chromosome. There are six possible ways to combine the 2 homologs contributed by one parent, hence the six means.

Chapter Two

Linkage mapping and QTL analysis of agronomic traits in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*)

Per H. McCord, Bryon R. Sosinski, Kathleen G. Haynes, Mark E. Clough, and G. Craig Yencho

Linkage mapping and QTL analysis of agronomic traits in tetraploid potato (*Solanum tuberosum* subsp. *tuberosum*)

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Abstract

Potato (*Solanum tuberosum* L) is one of the world's most important food crops, and has been the subject of a number of linkage mapping and QTL analyses over the years. However, most of this research has been done using diploid forms of potato, while commercial varieties are autotetraploid. Using a segregating tetraploid population, we developed a linkage map using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers, and searched for quantitative trait loci (QTL) via interval mapping and single-marker ANOVA. QTL were detected for flower color, foliage maturity, tuber skin texture, dry matter content, specific gravity, and yield. Most of the linkage groups were anchored to *Solanum* chromosomes using previously mapped SSR markers. Phenotypic data were collected over three seasons. The most significant QTL detected was for flower color. It was located on chromosome II and explained over 40% of the variation for this trait. This QTL most likely corresponds to the *R* locus responsible for red anthocyanin production. We also confirmed the presence of QTL for foliage maturity on chromosomes III and V, which had been detected in previous studies involving different populations. For skin texture, a trait that has not been previously mapped in potato, we detected a number of QTL. One QTL for texture, found on chromosome III of the female parent, explained more than 20% of the trait variation. By measuring specific gravity and dry matter independently we were able to detect QTL for these two traits that did not co-locate, even though the traits are strongly correlated. Most yield QTL were detected in one season only, which is probably a reflection of the variation between growing seasons and the complex nature of yield. Most QTL we detected accounted for 4.3-17.9% of observed variation. Many QTL could be modeled as simplex or duplex with dominant effects, but a large number displayed additive or inter-allelic interactive effects. Our mapping population segregates for a number of important agronomic traits, and the mapping and modeling of these traits could be improved by the use of more co-dominant markers, such as single nucleotide polymorphisms (SNPs).

Introduction

In crop plants, many traits of agronomic importance are quantitatively inherited. This is certainly true for potato (*Solanum tuberosum* L.), the world's fourth most important food crop (FAO 2007). Traits such as yield (Maris 1969), specific gravity (Freyre and Douches 1994), foliage maturity (Bradshaw et al. 2008), resistance to diseases such as powdery scab (reviewed in Harrison et al. 1997) and late blight (reviewed in Haynes et al. 1998), and resistance to insect pests (Flanders et al. 1992; Yencho et al. 1996) all show a continuous distribution. Many potato mapping and/or QTL analysis studies have been published during the course of more than two decades (Bonierbale et al. 1988; Gebhardt et al. 1989; Bonierbale et al. 1994; Freyre and Douches 1994; Schäffer-Pregl et al. 1998; Collins et al. 1999; Visker et al. 2005; Bradshaw et al. 2008; Khu et al. 2008). However, most of these projects have utilized diploid populations, while the vast majority of cultivated potatoes are autotetraploids (2n = 4x = 48). Autopolyploidy leads to polysomic inheritance, which is considerably more complex than disomic inheritance. Not only are larger population sizes required to accurately sample allelic combinations and model segregation ratios, the phenomenon of double reduction (Haynes and Douches 1994 and references therein) can skew observed recombination fractions, adversely affecting map distances. In addition, the modeling of genetic effects is more complex, as tri- and tetra-allelic interactions occur within loci. While much important and useful information has been obtained from QTL analysis at the diploid level, breeding for commercial cultivars occurs at the tetraploid level, and it would be advantageous to have better knowledge of the number, location, and effects of QTL in a tetraploid context. In this paper, we report our efforts to generate an anchored linkage map of tetraploid potato, and to identify and quantify QTL for flower color and five agronomic traits: dry matter content, specific gravity, foliage maturity, skin texture, and yield**.**

Materials and Methods

Population

Our mapping population, B2721, consisted of 160 individuals from a cross between an important chip processing cultivar, 'Atlantic' (Webb et al. 1978), and B1829-5, an advanced breeding selection from the USDA-ARS potato breeding program at Beltsville, MD. Progeny (20 genotypes) from this cross were first observed in 2004 and 2005 as part of a larger study of general combining ability for internal heat necrosis or IHN (Haynes, unpublished), another trait for which B2721 is segregating (McCord et al. in preparation). During 2005, minitubers generated from remnant seed from this cross were grown in the field as single plant plots spaced roughly 1 m apart in the row. The first 160 plants yielding enough tubers to produce at least 10 plants per genotype in the following year were collected to form B2721.

Phenotypic data collection

The phenotyping of B2721 began in 2006 when the population was planted in the field in an unreplicated trial with 6 plants per clone. In 2007 and 2008, the population was planted in a randomized complete block design with 2 replications and 10 plants per clone. The population was grown for evaluation each year at the North Carolina Department of Agriculture and Consumer Services, Tidewater Research Station (NCDA&CS TRS) in Plymouth, North Carolina. One hundred fifty-four clones were evaluated in 2007, and 156 were evaluated in 2008 (four individuals died, and two did not set sufficient seed for the 2007 season). Growing conditions (fertilizer and pesticides, planting and harvest dates, etc.) are described in detail in McCord et al. (in preparation).

Data for flower color and foliage maturity were collected during the growing season (2008 for flower color, 2007 and 2008 for foliage maturity). The range of flower colors was subdued in this population, and the trait was scored as '0' for white or '1' for lavender. Foliage maturity ratings were taken on each plot using a 6-point scale: 0 = 0-5% senescence; 1 = 10% yellowing of foliage; 2 = up to 25% yellow, vines wilting; 3 = 50% yellow, vines

wilting; 4 75-90% senesced, vines still green; 5 completely senesced. Plots were rated three times in 2007, on 13 June, 28 June, and 12 July. In 2008, maturity ratings were taken five times, on 9 June, 17 June, 25 June, 2 July, and 9 July. Ratings for each clone and date were averaged across plots, and the means were used to calculate the area under the "senescence curve" (AUSC) via the method of Shaner and Finney (1977) using Microsoft Excel.

Data for tuber traits were collected at harvest. Tubers were washed, and culled for excessive soft rot only (i.e. no culling for other diseases, sun scald, etc.). Both total yield and marketable yield (total – weight of culls) were calculated; subsequent analyses utilized the total yield data. Yield was calculated on a per-plant basis by dividing the total weight per plot by the number of plants per plot. All tubers (minus culls) in a plot were used to calculate the specific gravity (SG) via the following formula:

$$\mathbf{SG = (weight_{air} - weight_{water}) \div weight_{air}}$$

Tubers were scored for skin texture using a 4 point scale: 0 = smooth; 1 = slightly netted; 2 = netted; 3 = russet. Tubers were cut longitudinally into quarters for rating internal heat necrosis or IHN; analysis of IHN data, including QTL mapping, is being reported elsewhere (McCord et al. in preparation). For dry matter analysis, at least four tuber quarters (preferably from different tubers) were placed into plastic bags with wire closures and frozen at -20°C, lyophilized, and weighed. Clone means and trait correlations were calculated using the MEANS and CORR procedures of SAS (SAS, Cary, NC).

DNA extraction, marker generation and linkage map construction

The details of DNA extraction and marker generation are presented in McCord et al. (in preparation). We primarily used AFLP markers (Vos et al. 1995) to construct our linkage maps. SSR markers from tomato (Frary et al. 2005) and potato (Feingold et al. 2005) were included in order to anchor linkage groups to *Solanum* chromosomes.

Two linkage maps, one for each parent, were constructed using TetraploidMap (Hackett et al. 2007). This software package was developed for mapping in autotetraploid species, and was

designed to handle dominant markers in several dosage configurations, as well as codominant markers.

QTL analysis

We used a combination of interval mapping (IM) and single marker ANOVA to detect QTL. Both procedures are available in TetraploidMap (Hackett et al. 2007). Single marker ANOVA was only used in situations where IM could not be reliably applied. This was the case when only one homolog of a linkage group/chromosome could be identified, but also occurred for a few groups with multiple homologs. A p-value cutoff of 0.01 was used to declare a marker significant for a particular trait. This threshold was lowered to 0.05 in cases where the marker was significant (at $p < 0.05$) for that trait in at least one additional year. The IM routine of TetraploidMap first produces a graphical reconstruction of a linkage group (four homologs) of one parent, utilizing a branch and bound algorithm and the marker information from all progeny. Then, the conditional probabilities of each QTL genotype are calculated at 2 cM intervals. Phenotypic data are then regressed on the QTL genotypes at each position, with the regression coefficients being weighted by the conditional probabilities of the QTL genotypes. The full regression model considered by TetraploidMap includes 6 QTL genotypes, which is the number of possible combinations of the two alleles transmitted by one parent. In addition, the fit of ten simpler models (the six duplex genotypes considered singly, plus 4 simplex genotypes) are compared to the full model.

Permutation tests of at least 100 iterations were used to confirm the presence of a QTL; these tests were run on all traits with a LOD profile equal to or greater than 2.5, and on traits with LOD profiles that displayed a definite peak, but did not quite reach the 2.5 threshold. We also declared QTL present for traits with a LOD score of at least 1.8, *if* the trait had been detected in at least one other year via the permutation test. In some cases, LOD profiles suggested the presence of more than one QTL on a linkage group. Only the effects of the most significant QTL were calculated, as IM assumes only one QTL per chromosome. However, we reported the location and LOD score of all secondary QTL

above the 90% permutation threshold. The characteristics of QTL detected via interval mapping are listed in Table 4, while significant single markers are listed in Table 5. These results are also displayed graphically on the linkage maps in Figure 2.

Results

Trait distributions and correlations

As can be seen in the histograms in Figures 1a-f, all continuous traits displayed a more or less normal distribution. These traits also showed a good deal of transgressive segregation in that the trait values for each parent lay fairly close to the population mean, while many individuals displayed positive or negative transgression. Flower color (1f) was scored as a binary trait, and segregated in a ratio of 37 lavender: 85 white (38 clones did not bloom). Trait correlations are listed in Table 2. Foliage maturity was negatively correlated with yield and skin texture, and positively correlated with specific gravity. Specific gravity was positively correlated with dry matter content and had a weak but significant negative correlation with skin texture. Flower color was not correlated with any of the other traits.

Linkage maps

The map of 'Atlantic' consisted of 287 markers, 180 of which were simplex (segregating 1:1), 30 were duplex (segregating 5:1), 68 were double-simplex (segregating 3:1), and 9 were SSRs. The map of B1829-5 included 144 simplex, 26 duplex, 74 double simplex, and 10 SSR markers, for a total of 254. Thirty-two of the markers scored in 'Atlantic' and 25 markers scored in B1829-5 remained unlinked. Several of the SSRs produced banding patterns that were difficult to score as a codominant marker. In these cases, individual alleles were scored as dominant markers. SSR and double-simplex markers, which are present in both parents, were used to align the linkage groups where possible. This alignment was successful, with only one group from each parent remaining unaligned.

In addition, the use of these 'bridging' markers allowed smaller groups to be combined by virtue of their alignment to a single group from the other parent. Thirteen overall linkage groups were identified in 'Atlantic', and fourteen overall groups in B1829-5. In 'Atlantic', 10 of the linkage groups contained all four homologs, with the remaining 3 groups consisting of a single homolog (Table 2). Only five groups of B1829-5 contained all four homologs; the remaining linkage groups contained from one to three homologs (Table 2). Linkage groups from both parents were anchored to chromosomes I, II, III, IV, V, VI, VIII, and IX through the use of previously mapped SSR markers. In addition, one linkage group of B1829-5 was anchored to chromosome XI. Total map lengths (calculated from the lengths of the overall linkage groups) were 1051.8 cM for 'Atlantic', and 957.7 cM for B1829-5.

QTL analysis

Dry matter - In 'Atlantic', QTL for dry matter were detected by interval mapping on chromosomes II and III, and linkage groups VII and X. The QTL on chromosomes II and VII were detected in more than one year (Figure 2). Most of these QTL displayed additive or more complex gene action with the effect depending on the QTL alleles. The QTL that could be modeled as having a dominant effect were associated with lower dry matter (Table 3). One marker on chromosome VIII of 'Atlantic' was significantly associated with reduced dry matter content in 2006. In B1829-5, QTL for dry matter were found on chromosomes II, V, and VIII. QTL were detected on chromosome V in all three years of the study; the other QTL for dry matter were significant in one year only. Three of the QTL in B1829-5 were modeled as having dominant effects. The one on chromosome V in 2008 was associated with increased dry matter, while those on chromosome VIII in 2007 and chromosome II in 2008 were associated with reduced dry matter content. In addition to these intervals, two markers for increased dry matter were found on chromosome XI of B1829-5 (Table 5). The range of variation explained by all dry matter QTL in both parents (excluding single markers) was 4.5-17.9%.

Specific gravity (SG) - QTL for specific gravity were detected on chromosome II in at least two of three years in both parents (Figure 2 and Table 3). The QTL from both parents could generally be modeled as simplex dominant alleles, associated with higher SG. In 2008, a duplex dominant QTL for specific gravity was detected on chromosome V of B1829-5 and was associated with higher SG, while the QTL on B1829-5 chromosome VIII (2008 only) was associated with lower gravity. A single marker for increased gravity was found on chromosome IX of 'Atlantic' in 2006, and a group of ten linked markers on group XII of B1829-5 were associated with reduced specific gravity in 2008. Three double-simplex markers from group XIII were associated with lower specific gravity in two out of three years; being double-simplex markers, they were present in both parents. Most of the IM-detected QTL could be modeled as simplex alleles with dominant effects (Table 3). The variation explained by individual interval-mapped QTL for specific gravity ranged from 3.7-7.8%.

Maturity - As reported in McCord et al. (in preparation), QTL for foliage maturity were detected on 'Atlantic' chromosomes II, III, and V in both 2007 and 2008 (Figure 2 and Table 3). Although QTL were found on chromosome III in both years, they were in very different positions (over 80 cM apart), and had different effects. All QTL displayed dominant gene action. The QTL from chromosomes II, III (in 2008) and V were associated with later maturity (lower AUSC scores), while the QTL found on chromosome III in 2007 was associated with earlier maturity (higher AUSC scores). Variation explained by these QTL ranged from 8 to 14.3 percent. A single marker on chromosome IX, E45M58_101.7 was associated with later maturity in 2007. In B1829-5, a QTL was found at the same position on chromosome V in both years, explaining 9.6 percent of the variation in 2007, and 15.6 percent in 2008. This QTL displayed dominant gene action, and was associated with earlier maturity. One marker each was found on chromosome I, group X, and group XII. These markers were significant in 2007 and 2008, and all were associated with later foliage maturity.

Texture - In terms of interval-mapped QTL, we detected more regions associated with skin texture than any other trait (Figure 1 and Table 3). Dosages and effects of QTL (12 total) were nearly evenly split between those with dominant (seven) and additive gene action (five). We detected a minor QTL on chromosome VI of 'Atlantic' in all three years of the study, which explained 2.9-5.7 percent of variation depending on year. Although 'Atlantic' has netted skin, this QTL was associated with smoother skin. A QTL detected in 2 of 3 years on chromosome II was associated with netted skin, as were QTL on chromosomes III and V (both found only in 2008). In B1829-5, QTL for skin texture were detected in all three years on chromosome IX. They demonstrated additive gene action, and explained from 11.5 to 14.7 percent of the trait variation. On chromosome III, an additive QTL for skin texture was detected in 2007 only; it explained 20.7 percent of the phenotypic variation. Although they were both found on the same chromosome, the QTL from each parent on chromosome III do not appear to be allelic, as they are located approximately 40 cM from each other. The QTL on chromosome IX from each parent are closer together (2-22 cM, depending on the year). A single marker for increased netting was found on group XIV ('Atlantic') in 2008; no single markers were detected for skin texture in B1829-5.

Yield - One QTL for yield was detected in all three seasons (Figure 2 and Table 3). It was located on linkage group XII of 'Atlantic' and explained 7.9-9.3% of variation. In 2006 and 2008 a dominant model was not sufficient to fit the QTL, but it could be fitted in 2007, and was associated with increased yield. Four single markers were significantly associated with yield in 'Atlantic'; all were associated with reduced yield. In B1829-5, a QTL for yield was found in the same location on group VII in 2006 and 2007. The effect was minor (4.5-8.5%), and in 2007 could be modeled as having a dominant enhancing effect on yield. A dominant QTL for decreased yield was found on chromosome VI in 2006. QTL detected in B1829-5 chromosomes V (2006 only) and VIII (2008 only) were best modeled as having additive gene action. No single markers for yield were found in B1829-5.

Flower color - The single QTL with largest effect was for flower color, present as a simplex allele with dominant effect on chromosome II of 'Atlantic'. This QTL had a LOD score of 11.6, explained 41.1% of the variation, and was associated with pigmented flowers (Figure 2 and Table 3). A minor QTL explaining 8.6% of the variation was detected on linkage group XII of 'Atlantic'; it too was associated with pigmentation. In B1829-5 two QTL (one estimable) for flower color were detected. Both of these were in a different location from the 'Atlantic' QTL on chromosome II, and the estimable QTL was associated with decreased pigmentation.

Discussion

This research provides insight into the inheritance of several important traits in cultivated potato. It also contributes information important for relating the utilization of our QTL knowledge in diploid potatoes to applied breeding in cultivated autotetraploid potatoes, and underscores the need for additional QTL analyses in tetraploid potatoes.

In general, linkage mapping and QTL analysis in autotetraploids is more difficult compared to diploid species. Larger population sizes are required to adequately sample allelic combinations, and more markers are required to provide adequate coverage of the additional homologous chromosomes. In addition, tri- and tetra-allelic interactions can occur, a phenomenon not seen at the diploid level. Despite these challenges, it is worthwhile to map and model traits in a tetraploid context as information from diploid potatoes regarding important traits cannot account for these higher-order allelic interactions. In addition, the condition of polyploidy can cause changes in both genome organization (Song et al. 1995; Luo et al. 2004) and gene expression (Lu et al. 2006; Pan 2008) that would not be present at the diploid level. The results of our QTL mapping study demonstrate some of the complexities of tetraploid genetics. They also reinforce previous QTL mapping research using both tetraploids and diploids, and identify previously unreported QTL for traits of agronomic importance.

Mapping and modeling QTL in cultivated potato

This QTL mapping experiment demonstrated some of the complexities associated with mapping in the autotetraploid cultivated potato. Many of the QTL we detected could not be explained by a simplex or duplex dominant model. Consider, for example, the QTL for skin texture on chromosome III of B1829-5, which explained more than 20% of the variation for the trait. In Figure 4, it appears that genotype Q24 is significantly different than all other genotypes, suggesting a duplex dominant effect. In this case, the failure of such a model to be a statistically good fit to the data is probably due to poor marker density. Indeed, it can be seen in Figure 5 that only homolog 2 has markers along the length of the chromosome, and that homolog 3 contains only two markers. Better marker coverage would increase the power to detect the fit of simpler models in this example. This example is a good illustration of the additional number of markers required for good map coverage in tetraploid versus diploid potato; there are twice as many homologs to cover.

Another example illustrating the complexities associated with mapping in autopolyploids is illustrated in Figure 6 with the QTL for maturity detected on chromosome III of 'Atlantic' in 2007. Here, genotype Q34 contributes to a lower AUSC or later maturity date compared to the other possible genotypes. However, a duplex dominant model is not sufficient to explain the observed phenotype, because there are differences in the phenotypic means of the other five QTL genotypes. This suggests an additive model, which is more complex than that for a diploid due to the presence of more alleles. Additive and interactive (i.e. non-dominant) effects could probably be modeled more accurately by treating the homologs as separate chromosomes and performing multiple interval mapping (Kao et al. 1999), but this would require increased marker coverage across homologs. Even if the simple additive effects of various alleles were known in a diploid/disomic context, they may not accurately predict the effects in a tetraploid/tetrasomic background. These examples demonstrate the complexity encountered by working in a tetraploid system. In particular, the second example demonstrates the need for modeling traits in potato at the tetraploid level.

Trait correlations

The correlation between tuber dry matter content and specific gravity has long been known (LeClerg 1947), but in our case the correlation (r = 0.49 averaged over three years) was not as strong as that reported by LeClerg (r = 0.81-0.85) and others (Houghland 1966, r = 0.81); Schippers 1976, r = 0.91). This is due to the weak correlation observed between the two traits in 2008. The correlation was 0.67 in 2006, 0.79 in 2007, but only 0.32 in 2008. The cause of this reduction in the correlation between dry matter content and specific gravity is unknown. However, growing conditions in 2008 were sub-optimal, and because the population was clonally propagated each year to generate more seed, more clones appeared to be infected with virus each year. These conditions either separately or together could have adversely affected the relationship between the two traits. Despite the weakness of the correlation in 2008, there was little effect on the localization of QTL for dry matter in 2008; most were located near QTL for dry matter in 2006 and 2007. The more typical years, 2006 and 2007, still had correlations slightly below what other researchers have published, but this could well be due to genetic variation. Kushman and Haynes (1971) combined previous comparisons of specific gravity and dry matter to show that the relationship between the two can vary depending on the variety used; our population consisted of 160 clones or 'varieties'. This correlation can also vary between populations. A secondary mapping population (McCord et al. in preparation) was also phenotyped in 2007 and 2008; correlations between specific gravity and dry matter were 0.85 and 0.90 for the two years, respectively.

Although QTL for yield and foliage maturity were detected on some of the same chromosomes, the correlation between these traits is most likely physiological; clones that have a lower AUSC (later maturity) can take advantage of more of the growing season to increase their yield. The correlation between maturity and specific gravity can be at least partially explained by the colocalization of QTL for these traits in similar locations on chromosome V of 'Atlantic'. Since QTL for skin texture are also located on chromosome V

of ‘Atlantic’, the correlations between texture and maturity, and between texture and specific gravity are also probably due to this colocalization.

QTL—corroborations and new findings

As mentioned in the introduction, numerous QTL studies in potato have been performed over the years. This gave us a background of data to which we were able to compare our own QTL discoveries. A number of these earlier studies have detected major QTL for foliage maturity, a trait previously shown to be correlated with resistance to late blight (Collins et al. 1999). Collins et al. (1999), Visker et al. (2005), Malosetti et al. (2006), and Bradshaw et al. (2008) found major QTL for foliage maturity on chromosome V. Our experiments have confirmed these results as we also detected QTL for maturity on chromosome V in both parents of B2721, in both years that data were gathered for the trait (2007 and 2008). Visker et al. (2005) also detected a QTL for maturity on chromosome III, which was corroborated by our findings.

The most significant QTL detected in this study was for lavender flower color, on chromosome II of ‘Atlantic’. The *R* locus on chromosome II is involved in red anthocyanin production in potato flowers (van Eck et al. 1993). The *R* locus behaves in a dominant fashion, as did the QTL that we uncovered for flower color. Furthermore, the QTL fits a simplex model very well, and ‘Atlantic’ is known to be simplex for the *R* locus (De Jong et al. 2004). It is likely that the QTL we have detected is indeed the *R* locus. This hypothesis could be easily confirmed, as the gene for *R*, a dihydroflavonol 4-reductase, has recently been cloned (Zhang et al. submitted). A QTL for pigmented flowers was also located on chromosome II of B1829-5, but at a different position (34 vs. 8 cM). More extensive marker coverage may help refine the positions of these QTL, but it does not seem likely that they are allelic; the QTL from B1829-5 has a more subtle effect on flower color as compared to the QTL from ‘Atlantic’ (Table 3). Another QTL with a minor effect on flower color was found on linkage group XII of ‘Atlantic’.

Specific gravity, dry matter, and starch content are highly correlated traits (Table 2, see also LeClerg 1947, Houghland 1966). Therefore, one should expect linkage or co-localization of QTL for these traits. QTL for specific gravity were detected across multiple populations and environments on chromosomes I, II, V, and VII by Freyre and Douches (1994). We also detected QTL for specific gravity on chromosomes II and V in our mapping population. Bradshaw et al. (2008) estimated tuber dry matter content based on specific gravity measurements, and located a QTL on chromosome V as well. Schäffer-Pregl et al. (1998) estimated tuber starch content from specific gravity measurements in 2 diploid populations, one a cross between two dihaploid *S. tuberosum* individuals, the other a cross between a diploid *S. tuberosum* X *S. chacoense* and another dihaploid *S. tuberosum*. They detected 18 QTL for starch content, which were located on all 12 chromosomes. By measuring tuber dry matter both directly and indirectly, we followed a slightly different approach than Bradshaw et al. and Schäffer-Pregl et al. As a result, we were able to detect QTL for dry matter *per se* on chromosomes V and VIII in the same regions as QTL for specific gravity, but also QTL for dry matter on chromosome III, where no specific gravity QTL were found. Likewise, we detected significant single markers for specific gravity, but not dry matter, on chromosome IV.

Schäffer-Pregl et al. (1998) discovered yield QTL on chromosomes I, II, V, VI, VII, VIII, X, and XII. Bradshaw et al. (2008) also located QTL for yield on chromosomes I and VI, and Li et al. (2008) used association mapping to identify a minor QTL for yield on chromosome V. Our most consistent QTL for yield were on unanchored groups (VII and XII in our population), though we did detect yield QTL in single environments on chromosomes II and VIII. The high variability of yield QTL in our population is most likely due to the large year-to-year differences in growing conditions. Yields were much higher in 2006 than in 2007 or 2008, both of which had suboptimal rainfall (data not shown).

In addition to finding additional support for QTL already reported, we have discovered important regions not previously reported in the literature. Skin texture is an important trait in potato as a smooth skin is desirable for fresh-market varieties. Our population showed good segregation for skin texture, from smooth to russeted. In terms of explained variation, skin texture QTL were the most important of the QTL for agronomic traits. In particular, the QTL on chromosome II of 'Atlantic' and chromosome IX of B1829-5 are of interest due to their consistent detection and significant effect on the trait. A marker or markers for this trait would allow early screening of progeny for desirable skin texture, especially in a cross expected to segregate for the trait. This could make it more feasible to use a parent with undesirable skin texture, but with other desirable traits.

Conclusion

The findings we have presented, along with the recent work of Bradshaw et al. (2008), Khu et al. (2008), and Sagredo et al. (2006) have demonstrated both the challenges and the feasibility of studying potato genetics using tetraploid potatoes. We have provided corroborating evidence for the presence of foliage maturity QTL on chromosomes III and V, and QTL for specific gravity on chromosomes II and V. In addition, to our knowledge we have mapped the first known QTL for potato skin texture. With additional markers, it will be possible to more accurately model and select for valuable traits in this important crop at the tetraploid level. An effort to map and model traits in a tetraploid population using sequence-specific multi-allelic markers should be undertaken. This would be more efficient than using AFLP markers, which are random, less informative, and tend to cluster, even though they are less expensive on a per-marker basis. The PoMaMo (Potatoes, Maps, and More) database (Meyer et al. 2005) is a current source of potato SNPs, and is being augmented by SNP data from the SOLCAP project (Douches and De Jong 2009), with available data hosted at the Solanaceae Genomics Resource at Michigan State University (http://solanaceae.plantbiology.msu.edu/). Multi-allelic, PCR-based markers are available in

increasing numbers, and should permit the continuation and refinement of mapping and modeling important traits of potato at the tetraploid level.

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Table 1. Correlations for traits scored in B2721 (data averaged over three years). Flower color was not correlated with any other trait. Maturity = foliage maturity; SG = specific gravity; DM = dry matter; Texture = skin texture.

	Yield	SG	DM	Texture	Maturity
Yield		0.02	0.14	0.12	-0.45
		0.64	0.003	0.01	<.0001
SG			0.49	-0.23	0.49
			<.0001	<.0001	<.0001
DM				0.07	0.06
				0.12	0.28
Texture					-0.34
					<.0001

Table 2. List of linkage groups/chromosomes identified in each parent of B2721, and the number of homologs in each group.

Chromosome/Linkage Group	Number of homologs (Atlantic)	Number of homologs (B1829-5)
I	4	3
II	4	4
III	4	4
IV	4	3
V	4	3
VI	4	4
Group VII	4	4
VIII	1	2
IX	4	4
Group X	4	1
XI	0	2
Group XII	4	2
Group XIII	1	1
Group XIV	1	0
Group XV	0	1

Table 3. QTL detected in the B2721 population via interval mapping. Abbreviations: LG = linkage group/chromosome; ATL = 'Atlantic'; 1829-5 = B1829-5; % = percent of trait variation explained; yld = yield in grams per plant; sg = specific gravity; dm = dry matter; mat = foliage maturity; txt = skin texture; N.E = not estimable; QTL/no QTL = the mean of individuals with or without the detected QTL allele(s); SE = standard error. The two-digit number following each abbreviation refers to the season (2006-2008). LOD scores with an asterisk (*) were between the 90 and 95th percentile in a permutation test of at least 100 iterations. Scores with the ^ symbol were below the 90th percentile, but were at least 1.8, and were reported if a QTL was found in a similar location at least one other year, and met the more stringent LOD score and permutation test criteria.

Trait	LG	Parent	Position (cM)	LOD	%	Dosage/Effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
dm06	VII	ATL	70	2.34^	4.3	Additive		
dm06	III	ATL	98	3.53*	8	Additive		
dm06	II	ATL	92	2.39^	4.5	Additive		
dm06	V	1829-5	50	3.06*	6	Additive		
dm07	II	ATL	80	3.46*	9.8	Additive		
dm07	VII	ATL	64	5.1	17.9	Additive		
dm07	VIII	1829-5	0	2.47	7.8	Simplex, dominant	20.1 (0.1)	20.9 (0.1)
dm07	V	1829-5	40	2.99*	7.9	Additive		
dm08	X	ATL	82	2.57	9.3	Simplex, dominant	18.6 (0.4)	20.5 (0.4)
dm08	V	1829-5	0	2.2*	5.9	Simplex, dominant	20.4 (0.3)	18.8 (0.4)
dm08	II	1829-5	66	2.69	8.3	Duplex, dominant	19.5 (0.2)	22.5 (0.8)
sg07	II	ATL	88	3.32*	7.8	Additive		
sg07	II	1829-5	78	2.35	7.2	Simplex, dominant	1.077 (0.001)	1.073 (0.001)
sg08	II	ATL	88	2.38*	7.5	Simplex, dominant	1.073 (0.001)	1.068 (0.001)
sg08	VIII	1829-5	0	2.1	6.3	Simplex, dominant	1.068 (0.001)	1.073 (0.001)

Table 3- continued

Trait	LG	Parent	Position (cM)	LOD	%	Dosage/Effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
sg08	V	1829-5	56	2.36*	7.6	Duplex, dominant	1.066 (0.001)	1.072 (0.001)
sg08	II	1829-5	70	2.21*	6	Simplex, dominant	1.072 (0.001)	1.068 (0.001)
mat07	V	ATL	76	4.2	14.3	Duplex, dominant	71.6 (1.7)	90.7 (3.5)
mat07	III	ATL	88	2.6*	8.6	Duplex, dominant	78.4 (1.8)	62.5 (3.9)
mat07	II	ATL	38	1.91^	8	Simplex, dominant	66.3 (2.9)	78.7 (2.1)
mat07	V	1829-5	56	3.23	9.6	Duplex, dominant	77.6 (1.7)	61.5 (3.5)
mat08	V	ATL	74	3.83	13.1	Duplex, dominant	76.0 (2.6)	103.8 (5.3)
mat08	III	ATL	6	4.5	13.8	Simplex, dominant	94.0 (3.4)	71.4 (3.3)
mat08	II	ATL	36	2.33*	11.65	Simplex, dominant	66.9 (4.1)	88.8 (3.1)
mat08	V	1829-5	56	5.23	15.6	Duplex, dominant	87.1 (2.5)	57.0 (5.0)
txt06	VI	ATL	0	1.44*	3.8	Duplex, dominant	1.41 (0.06)	1.90 (0.17)
txt06	V	ATL	72	2.11*	6.8	Duplex, dominant	1.55 (0.06)	1.07 (0.13)
txt06	IX	1829-5	34	2.32	14.7	Additive		

Table 3- continued

Trait	LG	Parent	Position (cM)	LOD	%	Dosage/Effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
txt07	VI	ATL	0	1.94*	2.9	Additive		
txt07	V	ATL	34	2.23*	7.4	Simplex, dominant	0.98 (0.09)	1.43 0.10
txt07	II	ATL	88	4.56	14.5	Duplex, dominant	1.42 (0.08)	0.69 (0.13)
txt07	IX	1829-5	14	3.22	14.7	Additive		
txt07	III	1829-5	46	4.98	20.7	Additive		
txt08	VI	ATL	0	2	5.7	Duplex, dominant	1.14 (0.08)	1.95 (0.24)
txt08	III	ATL	0	3.62	11.9	Simplex, dominant	1.56 (0.11)	0.83 (0.13)
txt08	II	ATL	88	2.72*	8.5	Duplex, dominant	1.41 (0.10)	0.71 (0.17)
txt08	IX	1829-5	34	3.75	11.5	Additive		
yld06	XII	ATL	38	3.43	7.9	Additive		
yld06	VII	1829-5	62	2.31^	4.5	Additive		
yld06	VI	1829-5	6	2.05	6.1	Duplex, dominant	949 (27)	1167 (59)
yld06	V	1829-5	56	3.29*	6.7	Additive		
yld07	XII	ATL	48	2.76	8.4	Duplex, dominant	464 (12)	362 (24)
yld07	II	ATL	44	4.1	11.4	Additive		
yld07	VII	1829-5	62	2.52*	8.5	Duplex, dominant	421 (12)	529 (26)
yld08	XII	ATL	38	3.95	9.3	Additive		
yld08	VIII	1829-5	26	2.85	5.8	Additive		

Table 3- continued

Trait	LG	Parent	Position (cM)	LOD	%	Dosage/Effect	QTL Present Mean (SE)	QTL Absent Mean (SE)
Flower	XII	ATL	24	2.19	8.6	Simplex, dominant	0.42 (.05)	0.14 (0.06)
Flower	II	ATL	8	11.39	41.1	Simplex, dominant	0.62 (0.05)	0.02 (0.05)
Flower	II	1829-5	22	3.30	13.1	Duplex, dominant	0.22 (0.05)	0.66 (0.09)
Flower	II	1829-5	24	2.73	N.E.	N.E.		

Table 4. List of significant single markers detected by ANOVA. Markers were declared significant with a p-value < 0.01 for a single year, or < 0.05 for multiple years.

Trait	Marker	Parent	LG	p-value	Mean(0)	Mean(1)	SED
dm06	E45M53_511.8	ATL	VIII	0.004	20.9	20.0	0.3
dm06	E40M52_115.4	1829-5	XI	0.03	20.0	20.7	0.3
dm06	E42M54_123.5	1829-5	XI	0.007	19.6	20.6	0.4
dm07	E40M52_115.4	1829-5	XI	0.02	20.2	20.7	0.2
dm08	E40M52_115.4	1829-5	XI	0.04	19.1	20.1	0.5
sg06	E41M56_112.7	ATL	XIII	0.04	1.073	1.071	0.001
sg06	P12M42_171.3	ATL	XIII	0.01	1.074	1.071	0.001
sg06	P12M36_456.6	ATL	IX	0.009	1.070	1.073	0.001
sg06	E41M56_112.7	1829-5	XIII	0.04	1.073	1.071	0.001
sg06	P12M42_171.3	1829-5	XIII	0.01	1.074	1.071	0.001
sg06	E41M57_136.3	1829-5	XIII	0.03	1.073	1.071	0.0009
sg07	E41M56_112.7	ATL	XIII	0.04	1.077	1.074	0.001
sg07	P12M42_171.3	ATL	XIII	0.005	1.078	1.074	0.001
sg07	E45M52_100.0A	ATL	XIII	0.01	1.077	1.074	0.001
sg07	P12M42_171.3	1829-5	XIII	0.005	1.078	1.074	0.001
sg07	E41M57_136.3	1829-5	XIII	0.006	1.076	1.073	0.001
sg07	E45M52_100.0A	1829-5	XIII	0.01	1.077	1.074	0.001
sg07	E41M56_112.7	1829-5	XIII	0.04	1.077	1.074	0.001
sg08	E45M52_100.0A	ATL	XIII	0.04	1.072	1.070	0.001
sg08	E45M52_100.0A	1829-5	XIII	0.04	1.072	1.070	0.001

Table 4- continued

Trait	Marker	Parent	LG	p-value	Mean(0)	Mean(1)	SED
sg08	E32M41_486.3	1829-5	XII	0.006	1.074	1.069	0.001
sg08	E36M32_217.3	1829-5	XII	0.005	1.072	1.069	0.001
sg08	E38M54_258.5	1829-5	XII	0.002	1.072	1.069	0.001
sg08	E38M58_155.9	1829-5	XII	0.003	1.073	1.069	0.001
sg08	E40M59_100.0E	1829-5	XII	0.005	1.072	1.069	0.001
sg08	E44M52_332.1	1829-5	XII	0.003	1.073	1.069	0.001
sg08	E45M48_240.1	1829-5	XII	0.004	1.072	1.069	0.001
sg08	E44M52_332.1	1829-5	XII	0.003	1.073	1.069	0.001
sg08	E45M48_240.1	1829-5	XII	0.004	1.072	1.069	0.001
sg08	E45M53_197.1	1829-5	XII	0.005	1.072	1.069	0.001
sg08	E45M60_81.3	1829-5	XII	0.002	1.074	1.069	0.001
sg08	E38M56_173.3	1829-5	XII	0.00321	1.0724	1.0687	0.001
mat07	E45M58_101.7	ATL	IX	0.008	81.8	72.0	3.6
mat07	E45M60_81.3	1829-5	XII	0.02	81.6	72.6	3.9
mat08	E45M60_81.3	1829-5	XII	0.04	89.9	77.9	5.8
mat08	E38M54_235.4	1829-5	I	0.004	96.2	78.1	6.1
txt08	E38M54_202.6	ATL	XIV	0.006	0.91	1.39	0.17
yld06	E34M49_316.2	ATL	VI	0.004	1080 g	924 g	53 g
yld06	E34M52_347.9	ATL	VI	0.003	1100 g	933 g	55 g
yld06	E41M57_100.0-	ATL	VI	0.0008	1164 g	947 g	64 g
yld07	E36M60_287.8	ATL	VII	0.006	486 g	421 g	23 g

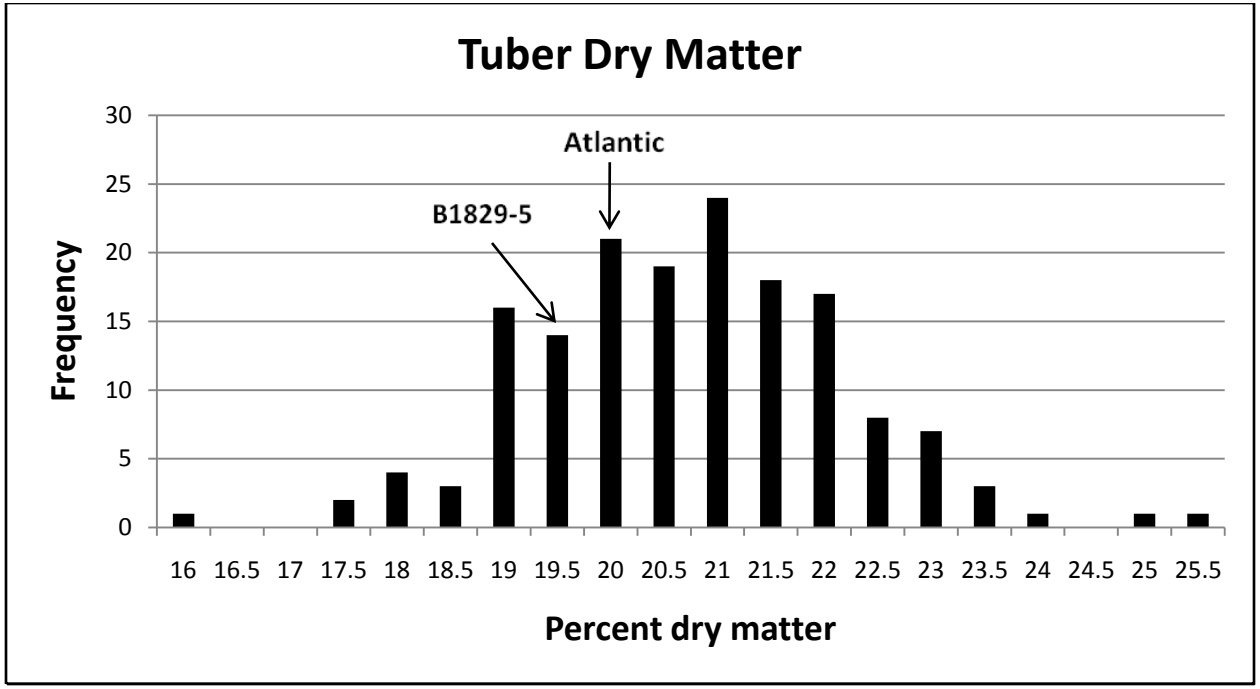


Figure 1a. Distribution of tuber dry matter content in B2721, averaged 2006-2008.

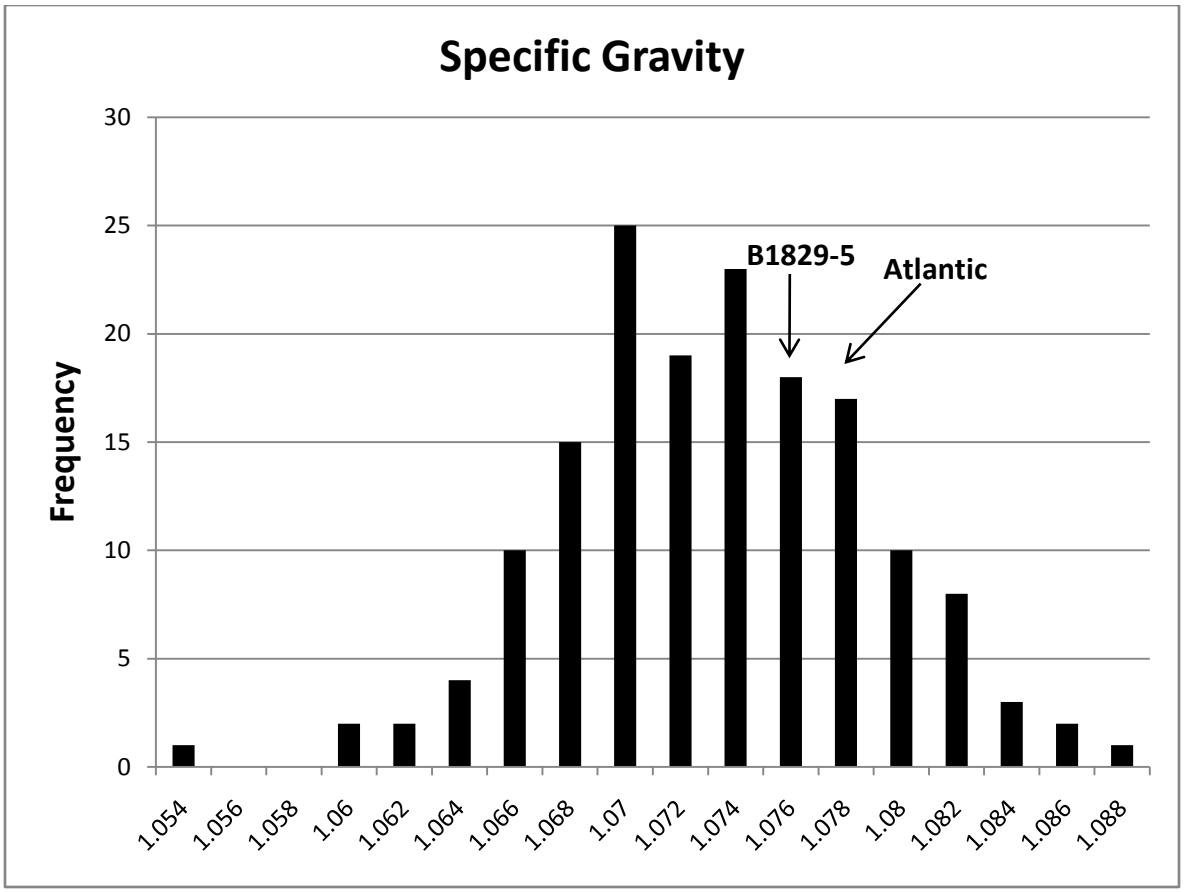


Figure 1b. Histogram of 3-year average specific gravity in B2721.

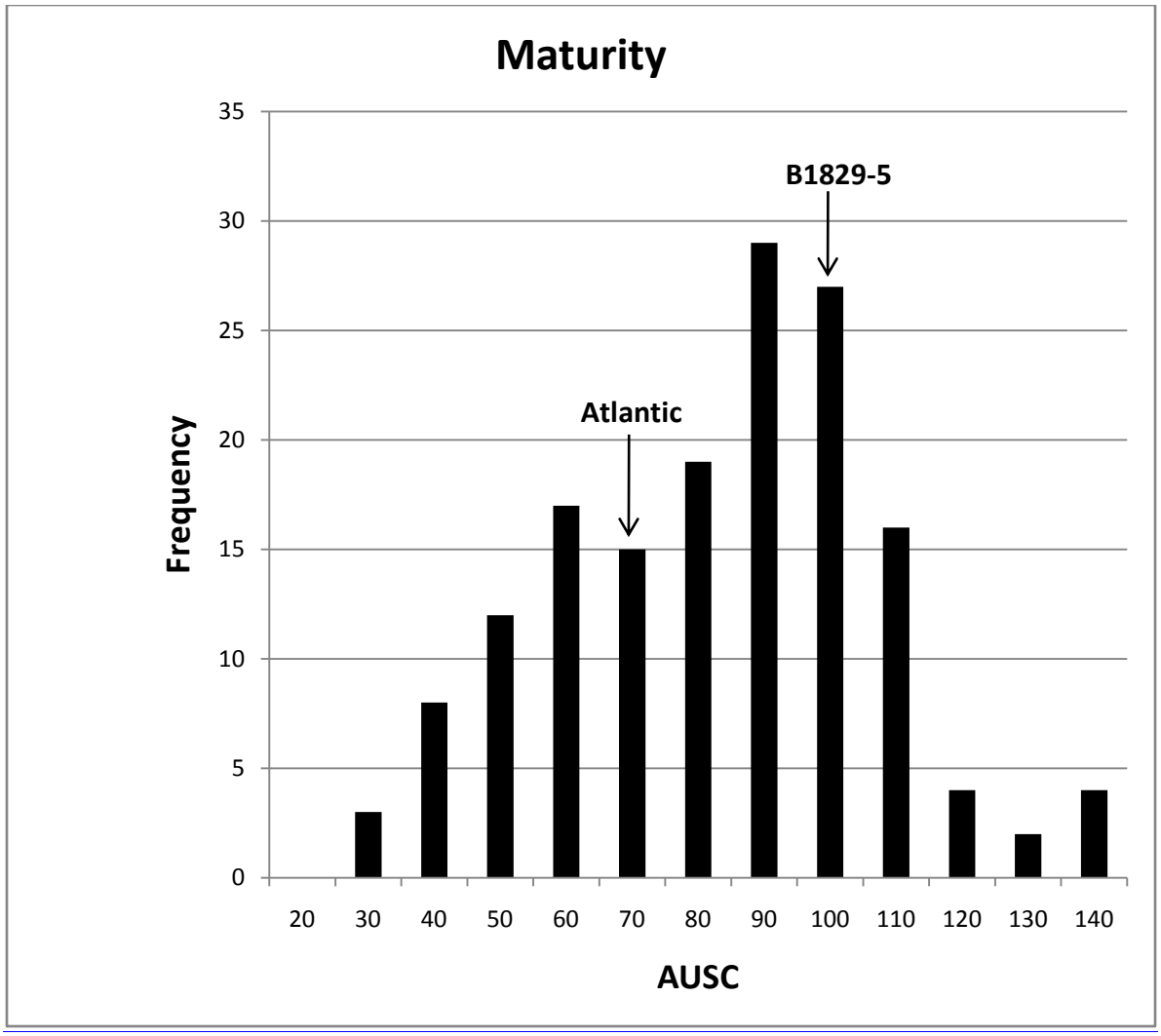


Figure 1c. Distribution of foliage maturity in B2721. Data were averaged over the 2007 and 2008 seasons. AUSC = Area Under the Senescence Curve. Columns with a higher AUSC display earlier foliage maturity.

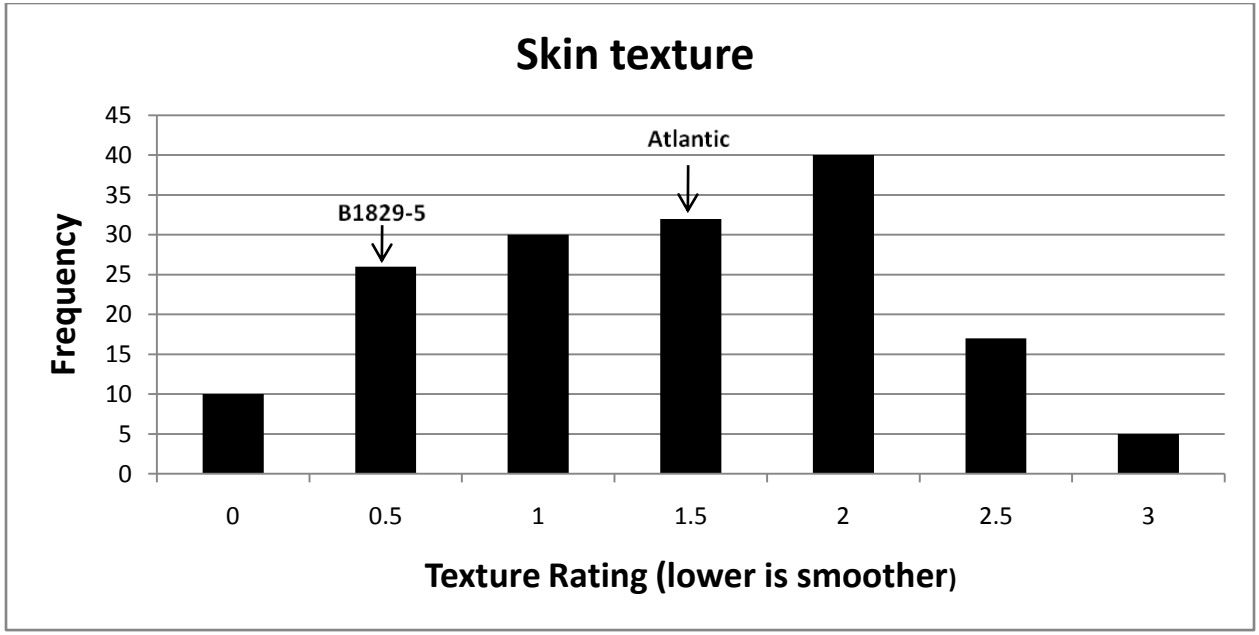


Figure 1d. Distribution of 3-year average skin texture rating of population B2721.

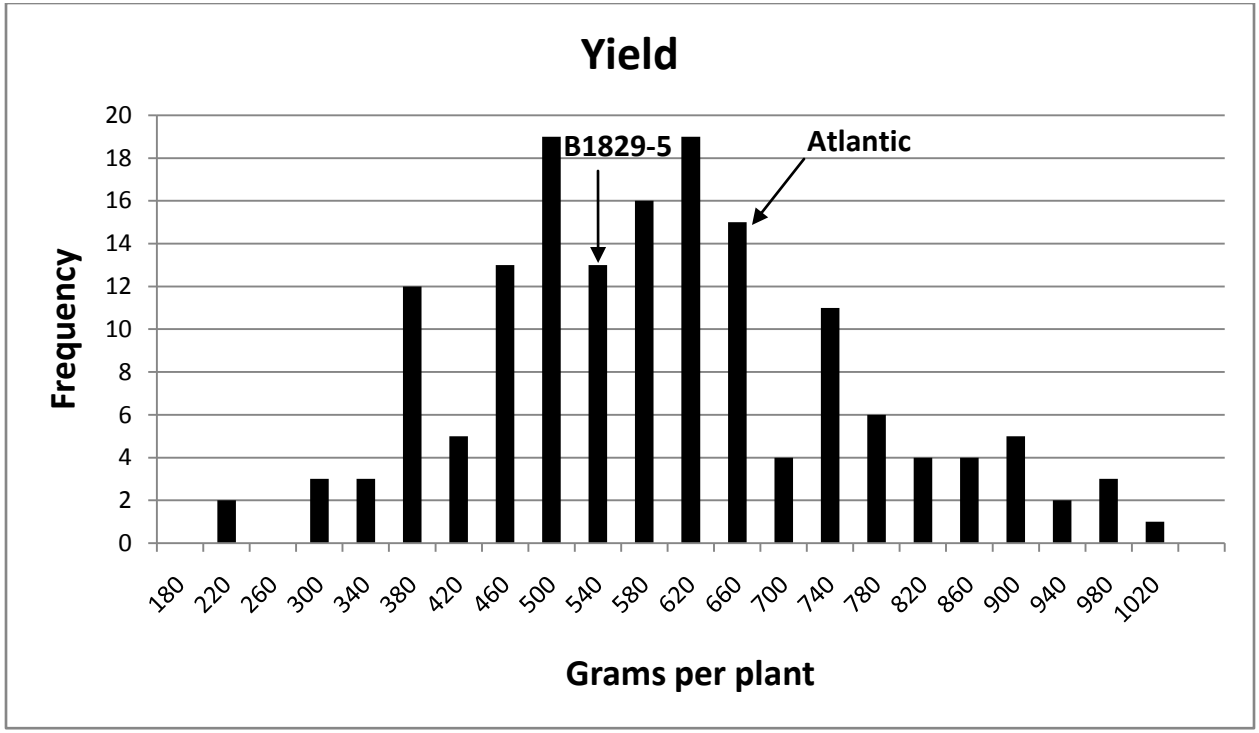


Figure 1e. Distribution of 3-year average yield of population B2721.

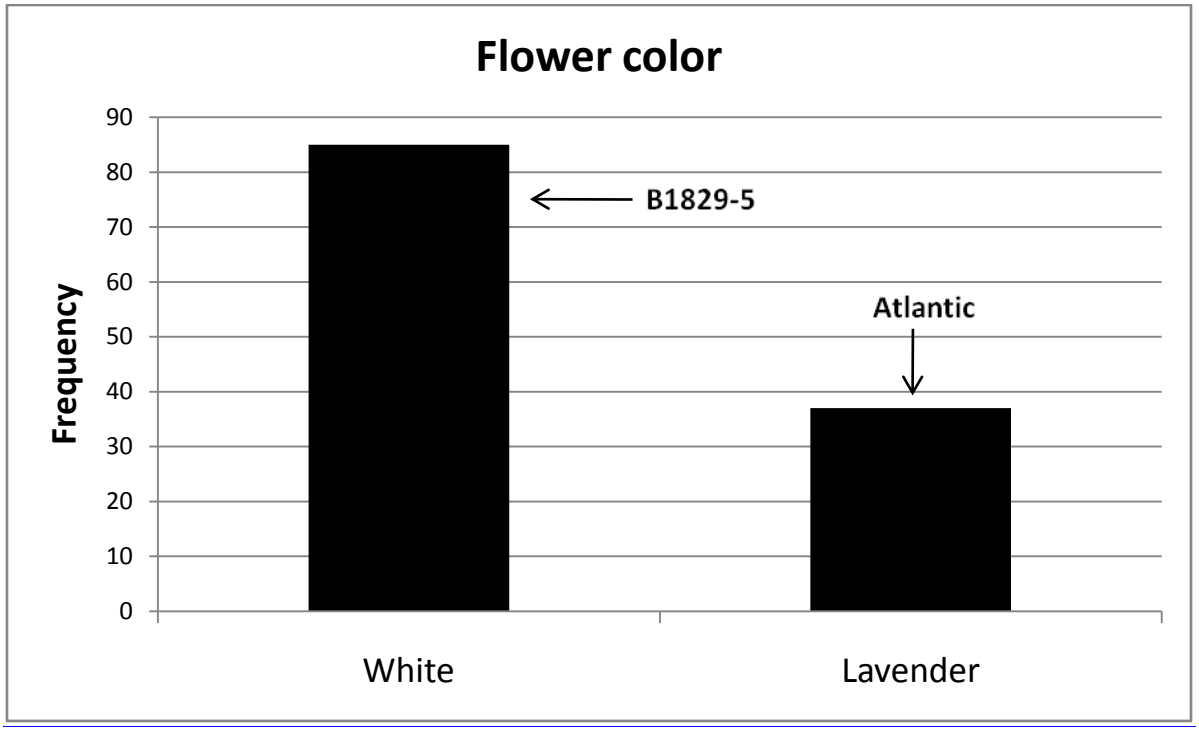
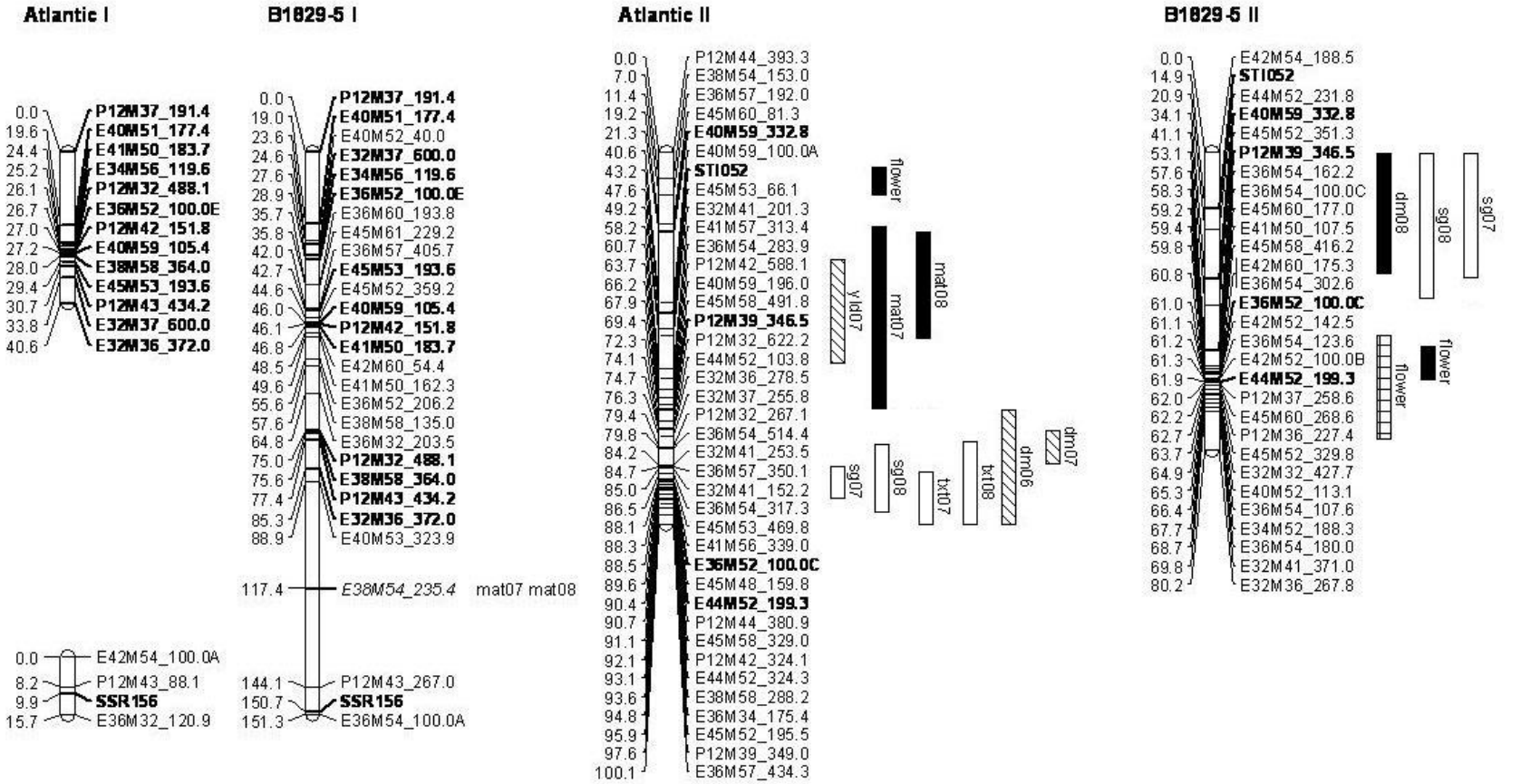


Figure 1f. Distribution of flower color in B2721.

Figure 2. Linkage maps of 'Atlantic' and B1829-5. Unless specified by the term 'group', Roman numerals refer to actual chromosomes. AFLP markers are denoted by the Keygene system of primer combinations (www.keygene.com/keygene/pdf/KF%20Primer%20enzyme%20combinations.pdf). The number after the underscore symbol is the approximate size in base pairs. Size estimates followed by letters refer to markers which were shorter than the shortest size marker measured, and are ranked alphabetically by size (A = largest, then B, and so forth). Tomato SSR markers are denoted by the prefix 'SSR', and potato SSR markers by the prefix 'STI'. Markers in bold type indicate SSR or double-simplex AFLP markers segregating in both parents, that were used to align the parental maps. Significant single markers are annotated by the trait(s) they are associated with, and are underlined if they affect the trait in a positive direction, or italicized if they have a negative effect. QTL are identified by shaded bars which represent a 1-LOD interval. Bar shading codes are the following: Black = dominant reducing effect; white= dominant enhancing effect; diagonal lines= complex additive effect; horizontal lines= non-estimable effect. Maps were drawn using MapChart (Voorrip 2002), with minor edits made using GIMP (www.gimp.org).


Atlantic I
B1829-5 I
Atlantic II
B1829-5 II
0.0 P12M37_191.4
19.6 E40M51_177.4
24.4 E41M50_183.7
25.2 E34M56_119.6
26.1 P12M32_488.1
26.7 E36M52_100.0E
27.0 P12M42_151.8
27.2 E40M59_105.4
28.0 E38M58_364.0
29.4 E45M53_193.6
30.7 P12M43_434.2
33.8 E32M37_600.0
40.6 E32M36_372.0
0.0 E42M54_100.0A
8.2 P12M43_88.1
9.9 SSR156
15.7 E36M32_120.9
0.0 P12M37_191.4
19.0 E40M51_177.4
23.6 E40M52_40.0
24.6 E32M37_600.0
27.6 E34M56_119.6
28.9 E36M52_100.0E
35.7 E36M60_193.8
35.8 E45M61_229.2
42.0 E36M57_405.7
42.7 E45M53_193.6
44.6 E45M52_359.2
46.0 E40M59_105.4
46.1 P12M42_151.8
46.8 E41M50_183.7
48.5 E42M60_54.4
49.6 E41M50_162.3
55.6 E36M52_206.2
57.6 E38M58_135.0
64.8 E36M32_203.5
75.0 P12M32_488.1
75.6 E38M58_364.0
77.4 P12M43_434.2
85.3 E32M36_372.0
88.9 E40M53_323.9
117.4 E38M54_235.4 mat07 mat08
144.1 P12M43_267.0
150.7 SSR156
151.3 E36M54_100.0A
0.0 P12M44_393.3
7.0 E38M54_153.0
11.4 E36M57_192.0
19.2 E45M60_81.3
21.3 E40M59_332.8
40.6 E40M59_100.0A
43.2 STI052
47.6 E45M53_66.1
49.2 E32M41_201.3
58.2 E41M57_313.4
60.7 E36M54_283.9
63.7 P12M42_588.1
66.2 E40M59_196.0
67.9 E45M58_491.8
69.4 P12M39_346.5
72.3 P12M32_622.2
74.1 E44M52_103.8
74.7 E32M36_278.5
76.3 E32M37_255.8
79.4 P12M32_267.1
79.8 E36M54_514.4
84.2 E32M41_253.5
84.7 E36M57_350.1
85.0 E32M41_152.2
86.5 E36M54_317.3
88.1 E45M53_469.8
88.3 E41M56_339.0
88.5 E36M52_100.0C
89.6 E45M48_159.8
90.4 E44M52_199.3
90.7 P12M44_380.9
91.1 E45M58_329.0
92.1 P12M42_324.1
93.1 E44M52_324.3
93.6 E38M58_288.2
94.8 E36M34_175.4
95.9 E45M52_195.5
97.6 P12M39_349.0
100.1 E36M57_434.3
flower
mat07
mat08
ytd07
sg07
sg08
txt07
txt08
drm06
drm07
0.0 E42M54_188.5
14.9 STI052
20.9 E44M52_231.8
34.1 E40M59_332.8
41.1 E45M52_351.3
53.1 P12M39_346.5
57.6 E36M54_162.2
58.3 E36M54_100.0C
59.2 E45M60_177.0
59.4 E41M50_107.5
59.8 E45M58_416.2
60.8 E42M60_175.3
E36M54_302.6
61.0 E36M52_100.0C
61.1 E42M52_142.5
61.2 E36M54_123.6
61.3 E42M52_100.0B
61.9 E44M52_199.3
62.0 P12M37_258.6
62.2 E45M60_268.6
62.7 P12M36_227.4
63.7 E45M52_329.8
64.9 E32M32_427.7
65.3 E40M52_113.1
66.4 E36M54_107.6
67.7 E34M52_188.3
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drm08
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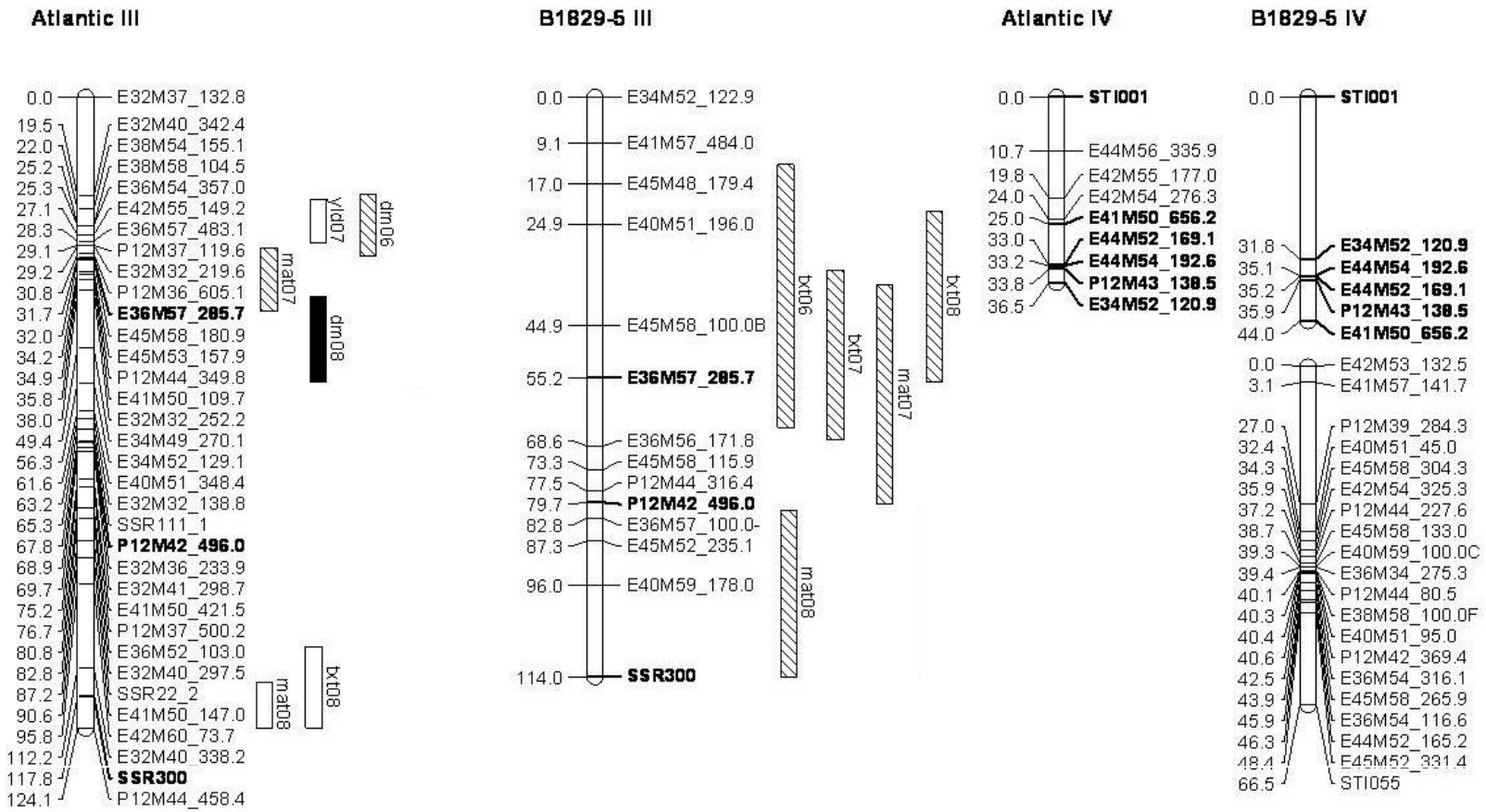
sg07
flower
flower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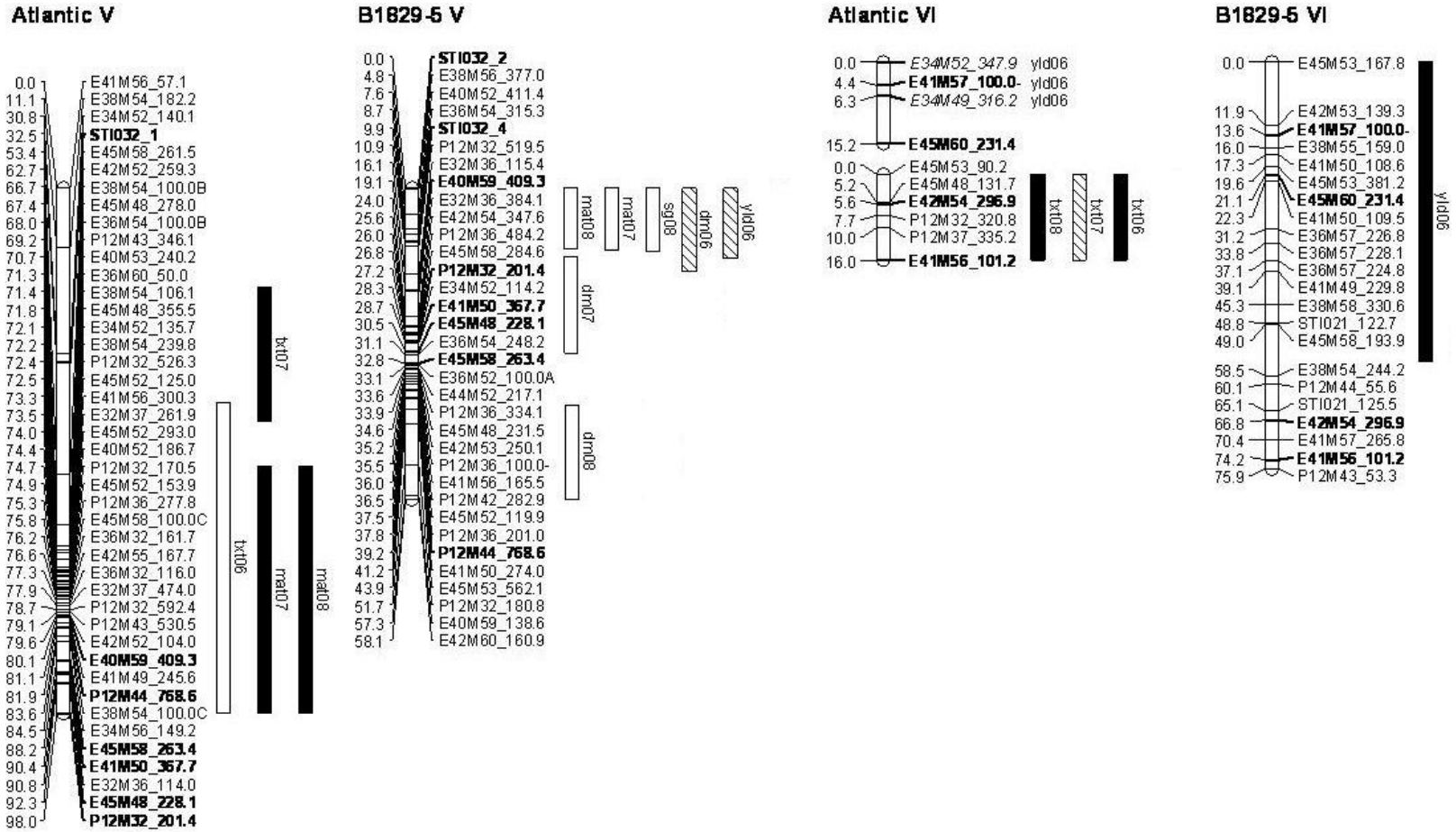
19.5 E32M40_342.4
22.0 E38M54_155.1
25.2 E38M58_104.5
25.3 E36M54_357.0
27.1 E42M55_149.2
28.3 E36M57_483.1
29.1 P12M37_119.6
29.2 E32M32_219.6
30.8 P12M36_605.1
31.7 E36M57_285.7
32.0 E45M58_180.9
34.2 E45M53_157.9
34.9 P12M44_349.8
35.8 E41M50_109.7
38.0 E32M32_252.2
49.4 E34M49_270.1
56.3 E34M52_129.1
61.6 E40M51_348.4
63.2 E32M32_138.8
65.3 SSR111_1
67.8 P12M42_496.0
68.9 E32M36_233.9
69.7 E32M41_298.7
75.2 E41M50_421.5
76.7 P12M37_500.2
80.8 E36M52_103.0
82.8 E32M40_297.5
87.2 SSR22_2
90.6 E41M50_147.0
95.8 E42M60_73.7
112.2 E32M40_338.2
117.8 SSR300
124.1 P12M44_458.4
vld07
dm06
mat07
dm08
mat08
bxt08
B1629-5 III
0.0 E34M52_122.9
9.1 E41M57_484.0
17.0 E45M48_179.4
24.9 E40M51_196.0
44.9 E45M58_100.0B
55.2 E36M57_285.7
68.6 E36M56_171.8
73.3 E45M58_115.9
77.5 P12M44_316.4
79.7 P12M42_496.0
82.8 E36M57_100.0-
87.3 E45M52_235.1
96.0 E40M59_178.0
114.0 SSR300
bxt06
bxt07
mat07
bxt08
mat08
Atlantic IV
0.0 STI001
10.7 E44M56_335.9
19.8 E42M55_177.0
24.0 E42M54_276.3
25.0 E41M50_656.2
33.0 E44M52_169.1
33.2 E44M54_192.6
33.8 P12M43_138.5
36.5 E34M52_120.9
B1629-5 IV
0.0 STI001
31.8 E34M52_120.9
35.1 E44M54_192.6
35.2 E44M52_169.1
35.9 P12M43_138.5
44.0 E41M50_656.2
0.0 E42M53_132.5
3.1 E41M57_141.7
27.0 P12M39_284.3
32.4 E40M51_45.0
34.3 E45M58_304.3
35.9 E42M54_325.3
37.2 P12M44_227.6
38.7 E45M58_133.0
39.3 E40M59_100.0C
39.4 E36M34_275.3
40.1 P12M44_80.5
40.3 E38M58_100.0F
40.4 E40M51_95.0
40.6 P12M42_369.4
42.5 E36M54_316.1
43.9 E45M58_265.9
45.9 E36M54_116.6
46.3 E44M52_165.2
48.4 E45M52_331.4
66.5 STI055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11.1 E38M54_182.2
30.8 E34M52_140.1
32.5 STI032_1
53.4 E45M58_261.5
62.7 E42M52_259.3
66.7 E38M54_100.0B
67.4 E45M48_278.0
68.0 E36M54_100.0B
69.2 P12M43_346.1
70.7 E40M53_240.2
71.3 E36M60_50.0
71.4 E38M54_106.1
71.8 E45M48_355.5
72.1 E34M52_135.7
72.2 E38M54_239.8
72.4 P12M32_526.3
72.5 E45M52_125.0
73.3 E41M56_300.3
73.5 E32M37_261.9
74.0 E45M52_293.0
74.4 E40M52_186.7
74.7 P12M32_170.5
74.9 E45M52_153.9
75.3 P12M36_277.8
75.8 E45M58_100.0C
76.2 E36M32_161.7
76.6 E42M55_167.7
77.3 E36M32_116.0
77.9 E32M37_474.0
78.7 P12M32_592.4
79.1 P12M43_530.5
79.6 E42M52_104.0
80.1 E40M59_409.3
81.1 E41M49_245.6
81.9 P12M44_768.6
83.6 E38M54_100.0C
84.5 E34M56_149.2
88.2 E45M58_263.4
90.4 E41M50_367.7
90.8 E32M36_114.0
92.3 E45M48_228.1
98.0 P12M32_201.4
txt07
txt06
mat07
mat08
B1629-5 V
0.0 STI032_2
4.8 E38M56_377.0
7.6 E40M52_411.4
8.7 E36M54_315.3
9.9 STI032_4
10.9 P12M32_519.5
16.1 E32M36_115.4
19.1 E40M59_409.3
24.0 E32M36_384.1
25.6 E42M54_347.6
26.0 P12M36_484.2
26.8 E45M58_284.6
27.2 P12M32_201.4
28.3 E34M52_114.2
28.7 E41M50_367.7
30.5 E45M48_228.1
31.1 E36M54_248.2
32.8 E45M58_263.4
33.1 E36M52_100.0A
33.6 E44M52_217.1
33.9 P12M36_334.1
34.6 E45M48_231.5
35.2 E42M53_250.1
35.5 P12M36_100.0-
36.0 E41M56_165.5
36.5 P12M42_282.9
37.5 E45M52_119.9
37.8 P12M36_201.0
39.2 P12M44_768.6
41.2 E41M50_274.0
43.9 E45M53_562.1
51.7 P12M32_180.8
57.3 E40M59_138.6
58.1 E42M60_160.9
mat08
dtm07
mat07
sg08
dtm06
dtm08
yld06
Atlantic VI
0.0 E34M52_347.9 yld06
4.4 E41M57_100.0- yld06
6.3 E34M49_316.2 yld06
15.2 E45M60_231.4
0.0 E45M53_90.2
5.2 E45M48_131.7
5.6 E42M54_296.9
7.7 P12M32_320.8
10.0 P12M37_335.2
16.0 E41M56_101.2
txt08
txt07
txt06
B1629-5 VI
0.0 E45M53_167.8
11.9 E42M53_139.3
13.6 E41M57_100.0-
16.0 E38M55_159.0
17.3 E41M50_108.6
19.6 E45M53_381.2
21.1 E45M60_231.4
22.3 E41M50_109.5
31.2 E36M57_226.8
33.8 E36M57_228.1
37.1 E36M57_224.8
39.1 E41M49_229.8
45.3 E38M58_330.6
48.8 STI021_122.7
49.0 E45M58_193.9
58.5 E38M54_244.2
60.1 P12M44_55.6
65.1 STI021_125.5
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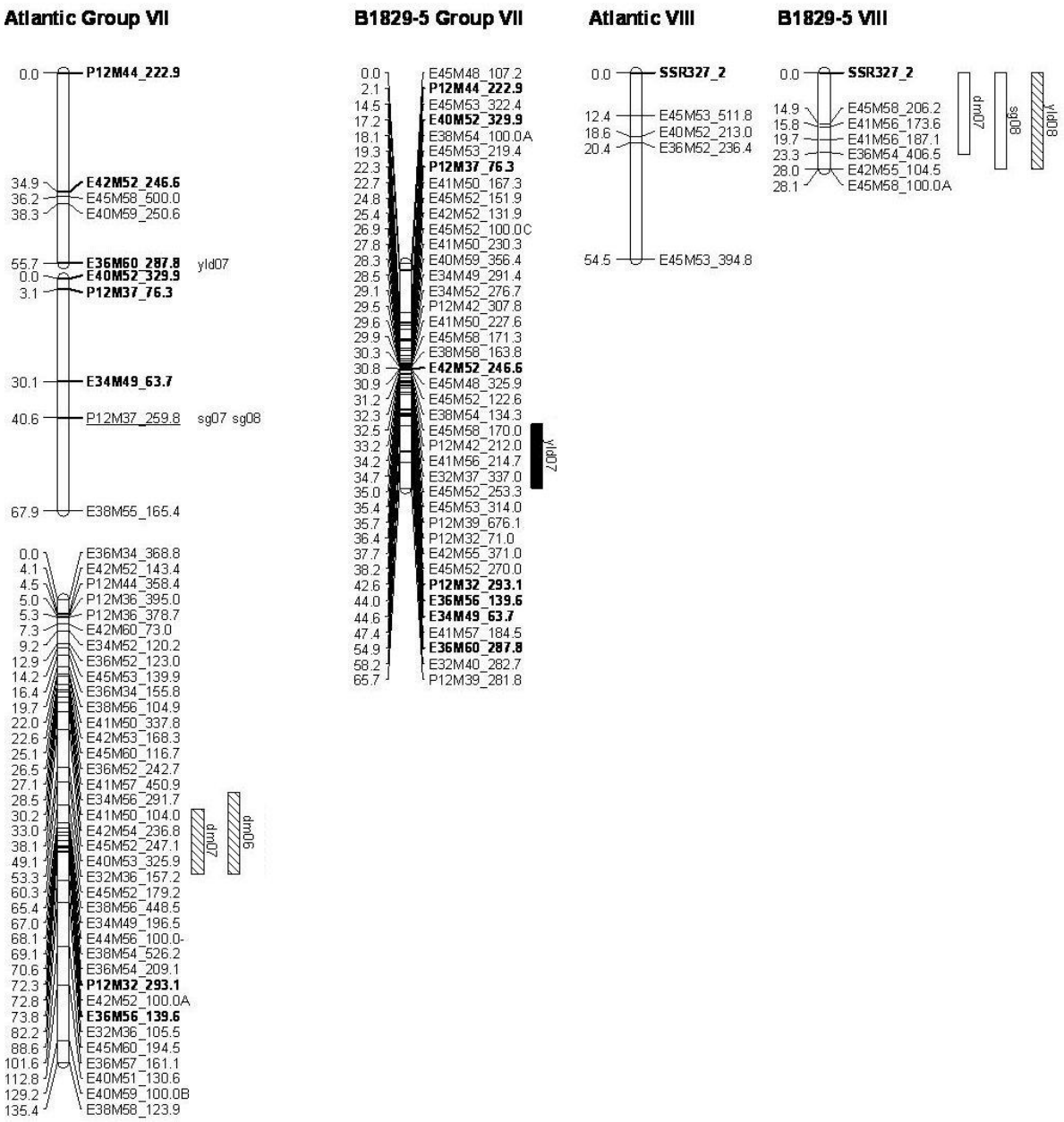
70.4 E41M57_265.8
74.2 E41M56_101.2
75.9 P12M43_53.3
yld06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34.9 E42M52_246.6
36.2 E45M58_500.0
38.3 E40M59_250.6
55.7 E36M60_287.8 yld07
0.0 E40M52_329.9
3.1 P12M37_76.3
30.1 E34M49_63.7
40.6 P12M37_259.8 sg07 sg08
67.9 E38M55_165.4
0.0 E36M34_368.8
4.1 E42M52_143.4
4.5 P12M44_358.4
5.0 P12M36_395.0
5.3 P12M36_378.7
7.3 E42M60_73.0
9.2 E34M52_120.2
12.9 E36M52_123.0
14.2 E45M53_139.9
16.4 E36M34_155.8
19.7 E38M56_104.9
22.0 E41M50_337.8
22.6 E42M53_168.3
25.1 E45M60_116.7
26.5 E36M52_242.7
27.1 E41M57_450.9
28.5 E34M56_291.7
30.2 E41M50_104.0
33.0 E42M54_236.8
38.1 E45M52_247.1
49.1 E40M53_325.9
53.3 E32M36_157.2
60.3 E45M52_179.2
65.4 E38M56_448.5
67.0 E34M49_196.5
68.1 E44M56_100.0-
69.1 E38M54_526.2
70.6 E36M54_209.1
72.3 P12M32_293.1
72.8 E42M52_100.0A
73.8 E36M56_139.6
82.2 E32M36_105.5
88.6 E45M60_194.5
101.6 E36M57_161.1
112.8 E40M51_130.6
129.2 E40M59_100.0B
135.4 E38M58_123.9
dm07
dm06
B1829-5 Group VII
0.0 E45M48_107.2
2.1 P12M44_222.9
14.5 E45M53_322.4
17.2 E40M52_329.9
18.1 E38M54_100.0A
19.3 E45M53_219.4
22.3 P12M37_76.3
22.7 E41M50_167.3
24.8 E45M52_151.9
25.4 E42M52_131.9
26.9 E45M52_100.0C
27.8 E41M50_230.3
28.3 E40M59_356.4
28.5 E34M49_291.4
29.1 E34M52_276.7
29.5 P12M42_307.8
29.6 E41M50_227.6
29.9 E45M58_171.3
30.3 E38M58_163.8
30.8 E42M52_246.6
30.9 E45M48_325.9
31.2 E45M52_122.6
32.3 E38M54_134.3
32.5 E45M58_170.0
33.2 P12M42_212.0
34.2 E41M56_214.7
34.7 E32M37_337.0
35.0 E45M52_253.3
35.4 E45M53_314.0
35.7 P12M39_676.1
36.4 P12M32_71.0
37.7 E42M55_371.0
38.2 E45M52_270.0
42.6 P12M32_293.1
44.0 E36M56_139.6
44.6 E34M49_63.7
47.4 E41M57_184.5
54.9 E36M60_287.8
58.2 E32M40_282.7
65.7 P12M39_281.8
yld07
Atlantic VIII
0.0 SSR327_2
12.4 E45M53_511.8
18.6 E40M52_213.0
20.4 E36M52_236.4
54.5 E45M53_394.8
B1829-5 VIII
0.0 SSR327_2
14.9 E45M58_206.2
15.8 E41M56_173.6
19.7 E41M56_187.1
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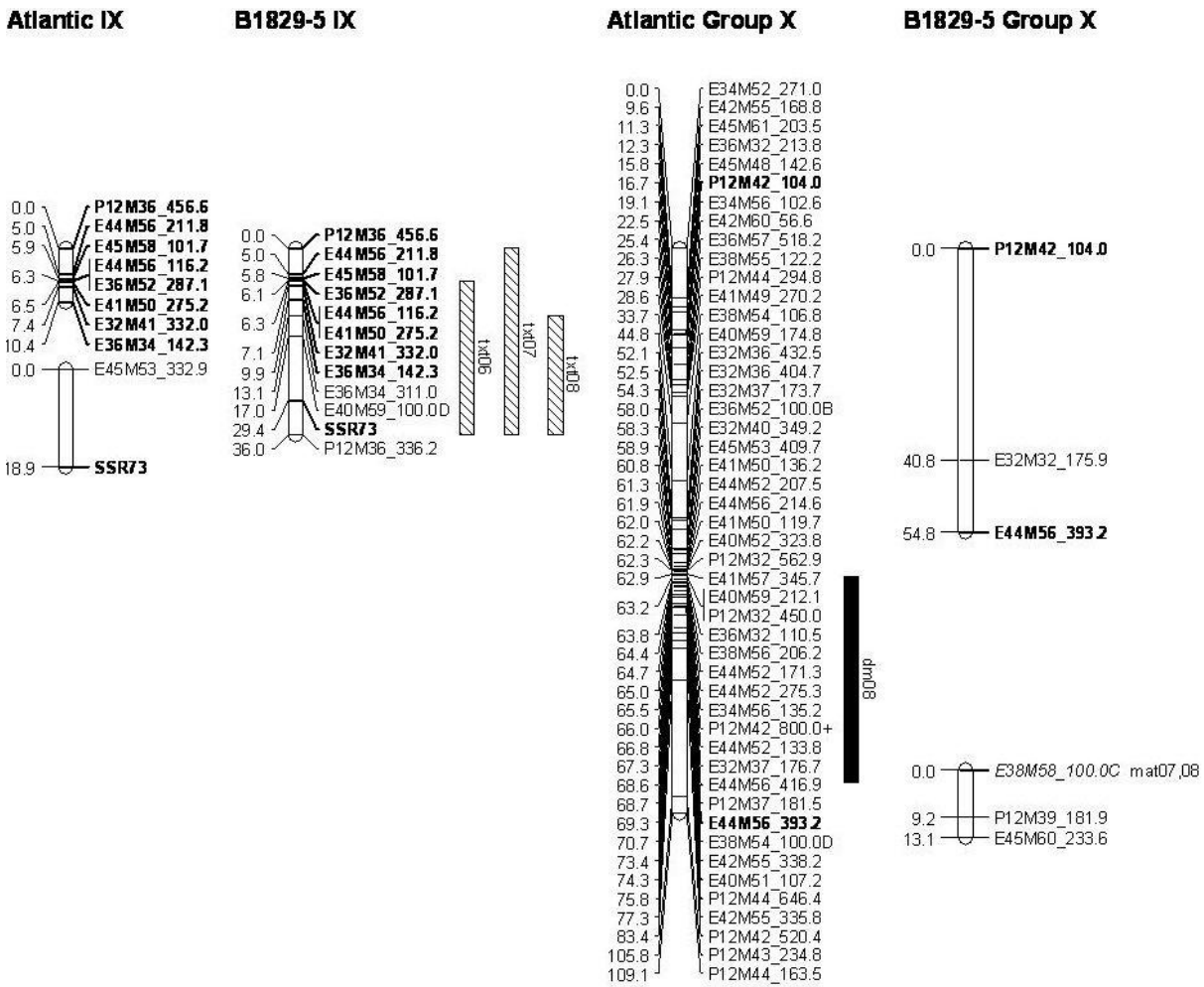
28.0 E42M55_104.5
28.1 E45M58_100.0A
dm07
sg08
yld08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Atlantic Group X
B1829-5 Group X
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5.9 E45M58_101.7
6.3 E44M56_116.2
E36M52_287.1
6.5 E41M50_275.2
7.4 E32M41_332.0
10.4 E36M34_142.3
0.0 E45M53_332.9
18.9 SSR73
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5.0 E44M56_211.8
5.8 E45M58_101.7
6.1 E36M52_287.1
6.3 E44M56_116.2
E41M50_275.2
7.1 E32M41_332.0
9.9 E36M34_142.3
13.1 E36M34_311.0
17.0 E40M59_100.0D
29.4 SSR73
36.0 P12M36_336.2
1x06
1x07
1x08
0.0 E34M52_271.0
9.6 E42M55_168.8
11.3 E45M61_203.5
12.3 E36M32_213.8
15.8 E45M48_142.6
16.7 P12M42_104.0
19.1 E34M56_102.6
22.5 E42M60_56.6
25.4 E36M57_518.2
26.3 E38M55_122.2
27.9 P12M44_294.8
28.6 E41M49_270.2
33.7 E38M54_106.8
44.8 E40M59_174.8
52.1 E32M36_432.5
52.5 E32M36_404.7
54.3 E32M37_173.7
58.0 E36M52_100.0B
58.3 E32M40_349.2
58.9 E45M53_409.7
60.8 E41M50_136.2
61.3 E44M52_207.5
61.9 E44M56_214.6
62.0 E41M50_119.7
62.2 E40M52_323.8
62.3 P12M32_562.9
62.9 E41M57_345.7
63.2 E40M59_212.1
P12M32_450.0
63.8 E36M32_110.5
64.4 E38M56_206.2
64.7 E44M52_171.3
65.0 E44M52_275.3
65.5 E34M56_135.2
66.0 P12M42_800.0+
66.8 E44M52_133.8
67.3 E32M37_176.7
68.6 E44M56_416.9
68.7 P12M37_181.5
69.3 E44M56_393.2
70.7 E38M54_100.0D
73.4 E42M55_338.2
74.3 E40M51_107.2
75.8 P12M44_646.4
77.3 E42M55_335.8
83.4 P12M42_520.4
105.8 P12M43_234.8
109.1 P12M44_163.5
dm08
0.0 P12M42_104.0
40.8 E32M32_175.9
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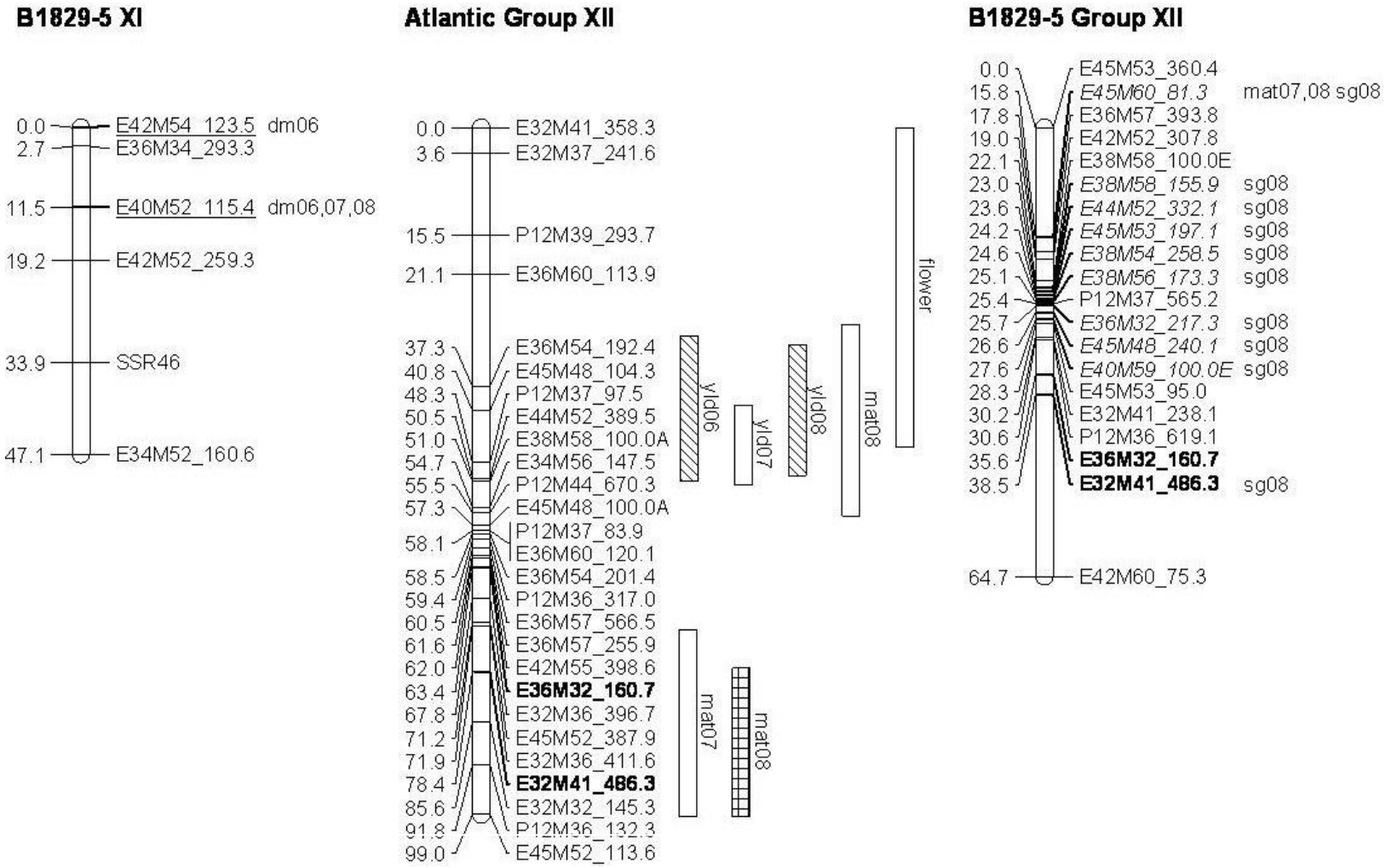
0.0 E38M58_100.0C mat07,08
9.2 P12M39_181.9
13.1 E45M60_233.6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2.7 E36M34_293.3
11.5 E40M52_115.4 dm06,07,08
19.2 E42M52_259.3
33.9 SSR46
47.1 E34M52_160.6
Atlantic Group XII
0.0 E32M41_358.3
3.6 E32M37_241.6
15.5 P12M39_293.7
21.1 E36M60_113.9
37.3 E36M54_192.4
40.8 E45M48_104.3
48.3 P12M37_97.5
50.5 E44M52_389.5
51.0 E38M58_100.0A
54.7 E34M56_147.5
55.5 P12M44_670.3
57.3 E45M48_100.0A
58.1 P12M37_83.9
E36M60_120.1
58.5 E36M54_201.4
59.4 P12M36_317.0
60.5 E36M57_566.5
61.6 E36M57_255.9
62.0 E42M55_398.6
63.4 E36M32_160.7
67.8 E32M36_396.7
71.2 E45M52_387.9
71.9 E32M36_411.6
78.4 E32M41_486.3
85.6 E32M32_145.3
91.8 P12M36_132.3
99.0 E45M52_113.6
yld06
yld07
yld08
mat08
flower
mat07
mat08
B1829-5 Group XII
0.0 E45M53_360.4
15.8 E45M60_81.3 mat07,08 sg08
17.8 E36M57_393.8
19.0 E42M52_307.8
22.1 E38M58_100.0E
23.0 E38M58_155.9 sg08
23.6 E44M52_332.1 sg08
24.2 E45M53_197.1 sg08
24.6 E38M54_258.5 sg08
25.1 E38M56_173.3 sg08
25.4 P12M37_565.2
25.7 E36M32_217.3 sg08
26.6 E45M48_240.1 sg08
27.6 E40M59_100.0E sg08
28.3 E45M53_95.0
30.2 E32M41_238.1
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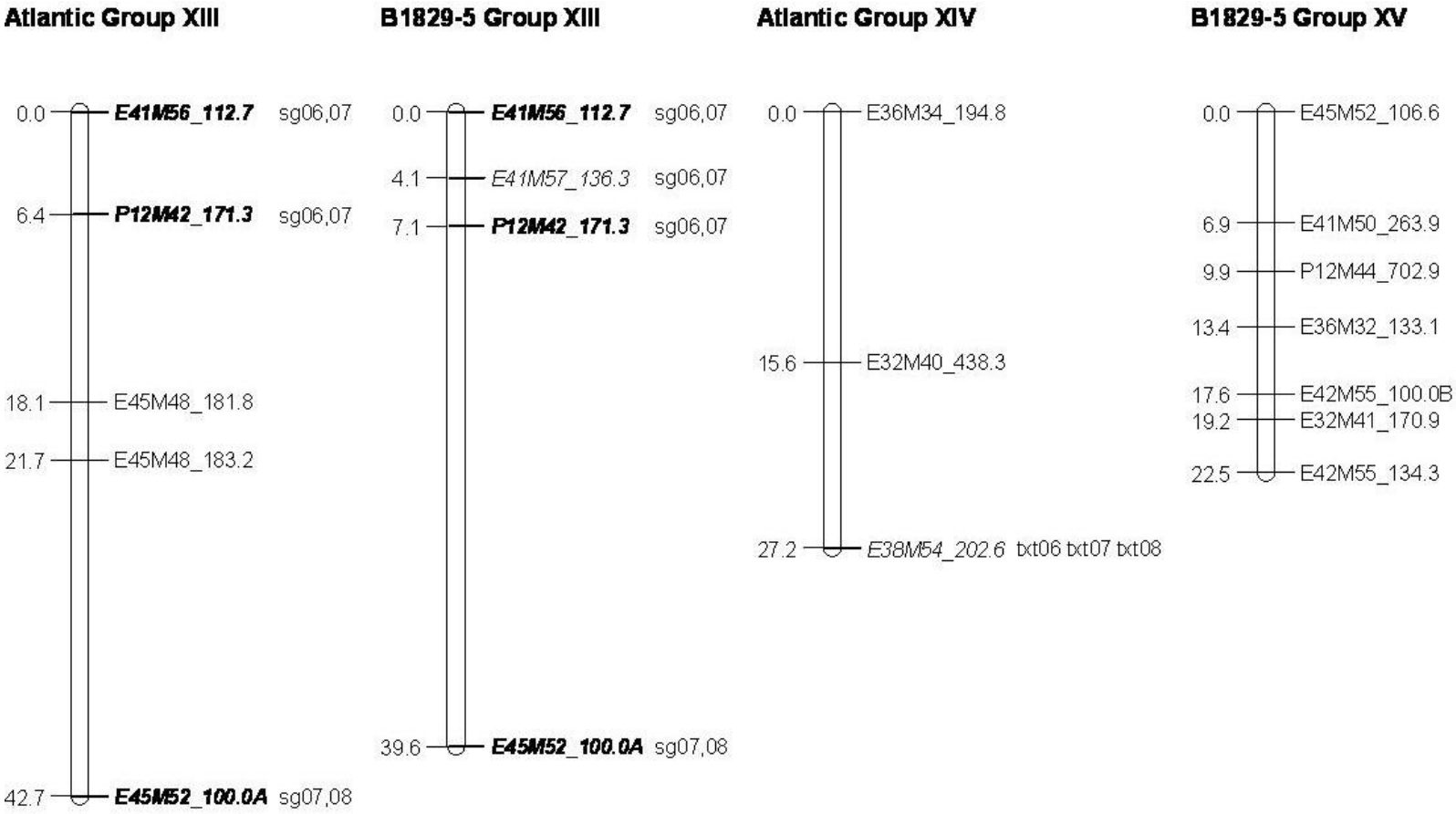
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6.4 P12M42_171.3 sg06,07
18.1 E45M48_181.8
21.7 E45M48_183.2
42.7 E45M52_100.0A sg07,08
B1829-5 Group XIII
0.0 E41M56_112.7 sg06,07
4.1 E41M57_136.3 sg06,07
7.1 P12M42_171.3 sg06,07
39.6 E45M52_100.0A sg07,08
Atlantic Group XIV
0.0 E36M34_194.8
15.6 E32M40_438.3
27.2 E38M54_202.6 txt06 txt07 txt08
B1829-5 Group XV
0.0 E45M52_106.6
6.9 E41M50_263.9
9.9 P12M44_702.9
13.4 E36M32_133.1
17.6 E42M55_100.0B
19.2 E32M41_170.9
22.5 E42M55_134.3

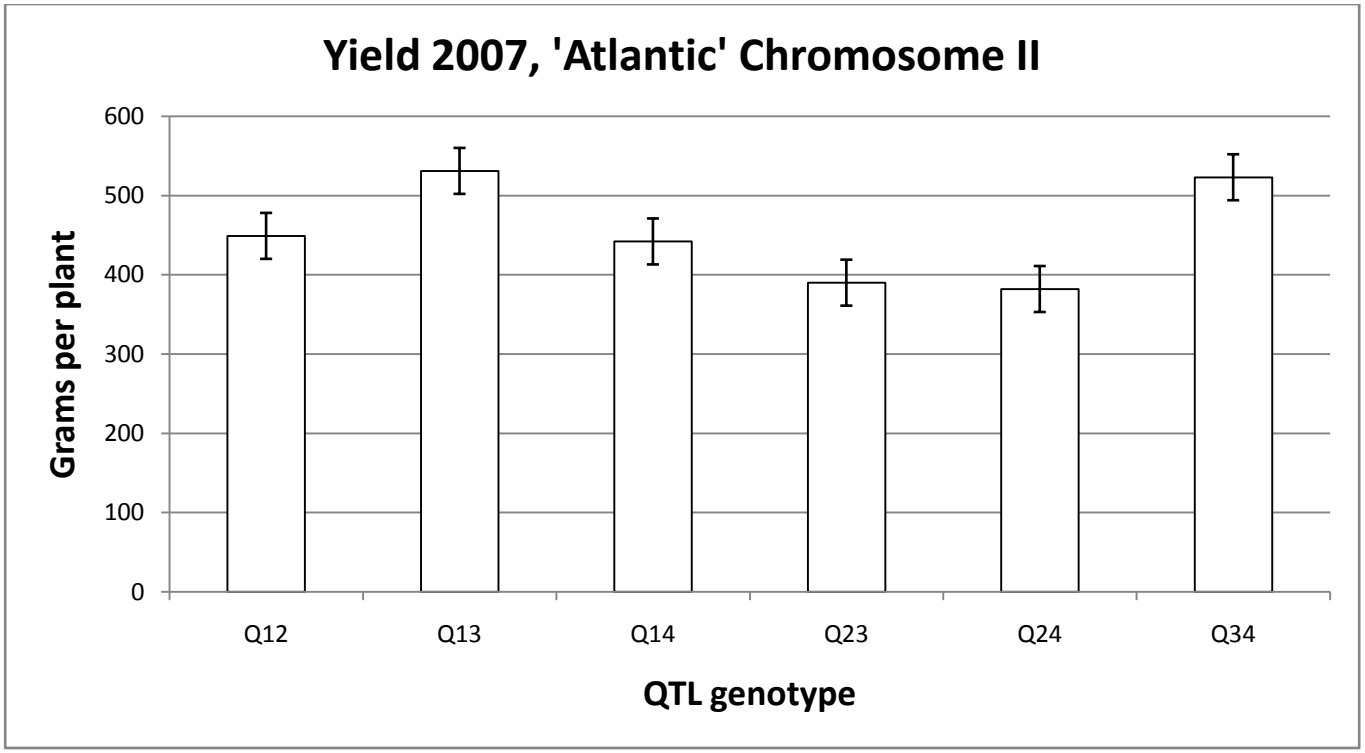

Figure 3. Allelic effects of the QTL for *yld07* on chromosome II of Atlantic. Error bars are the average of the standard errors of all genotypes. 'Q' refers to the putative QTL; 1-4 refer to the four homologs of each chromosome. There are six possible ways to combine the 2 homologs contributed by one parent, hence the six means.

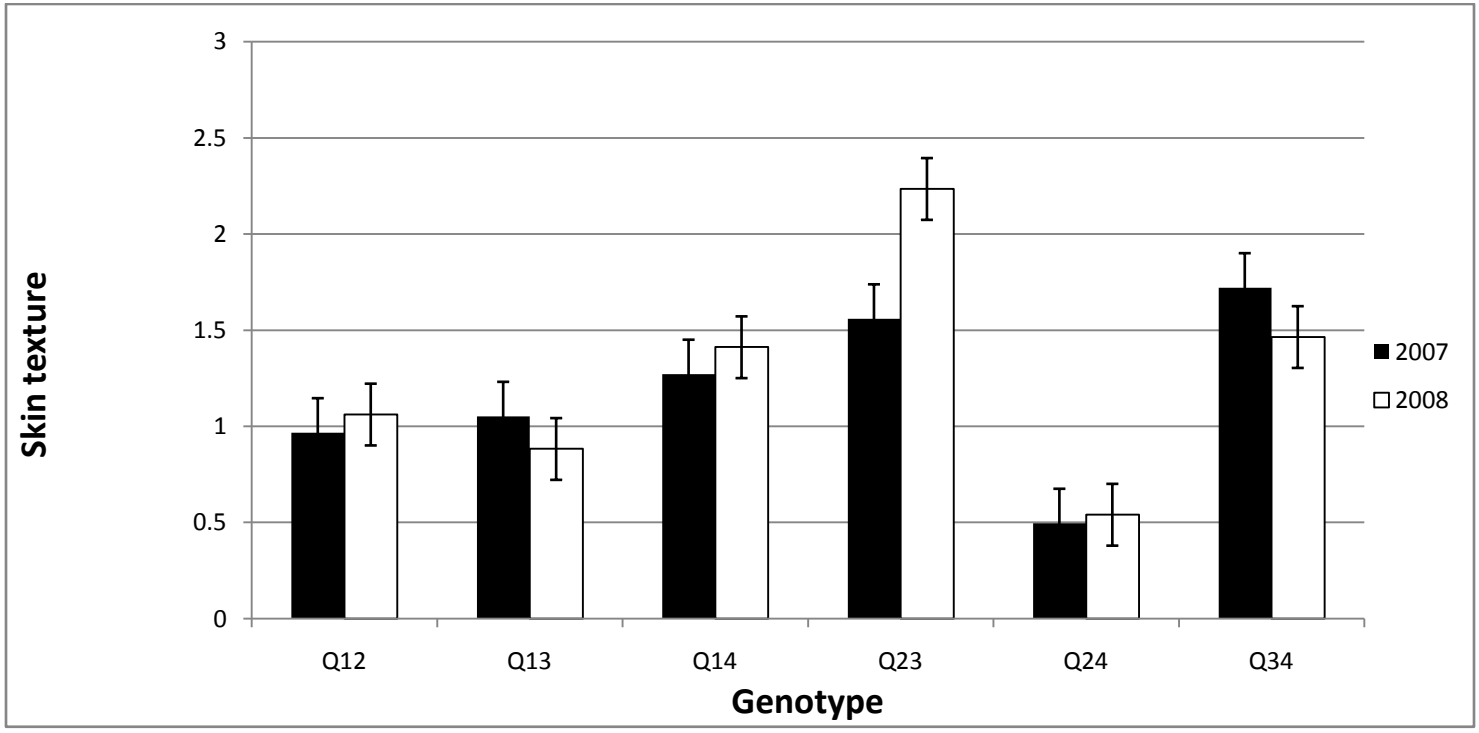

Figure 4. Allelic effects on skin texture of the QTL on chromosome III of B1829-5. A higher skin texture rating indicates rougher (more netted) skin. Error bars are the average of the standard errors of all genotypes across both years.

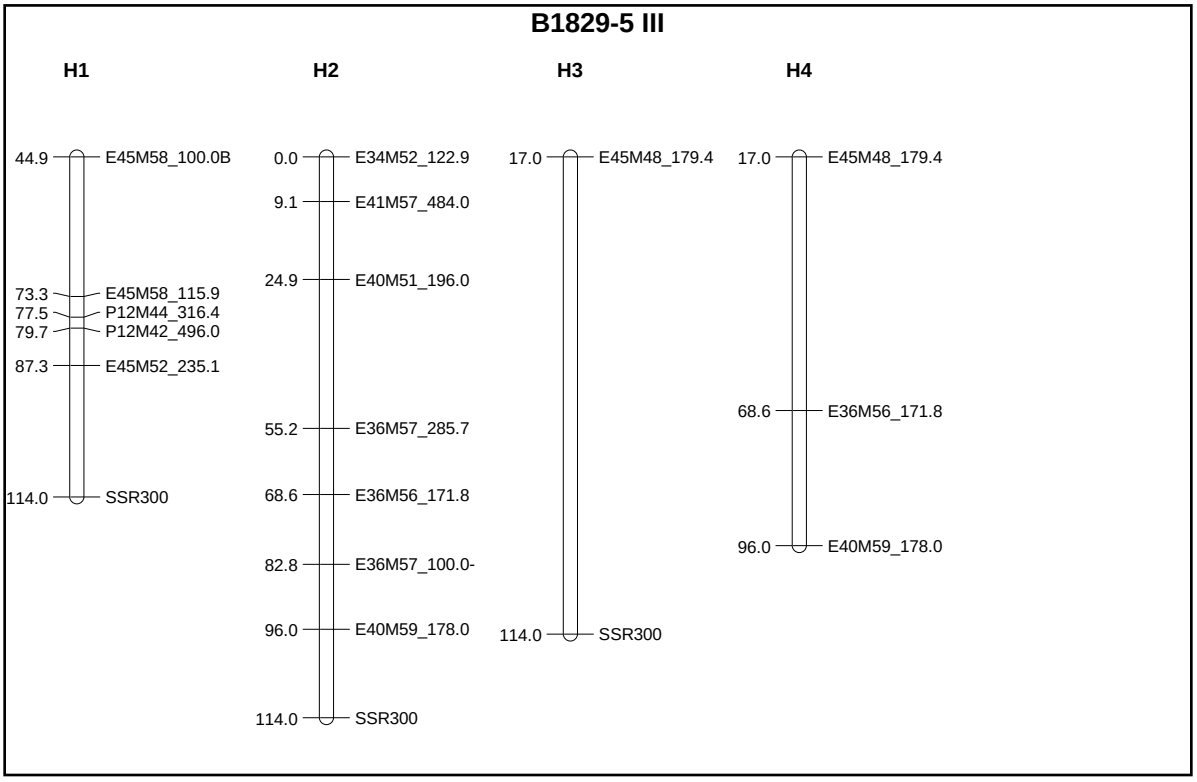

Figure 5. Expanded view of chromosome III from B1829-5, showing marker density on each homolog (H1-H4).

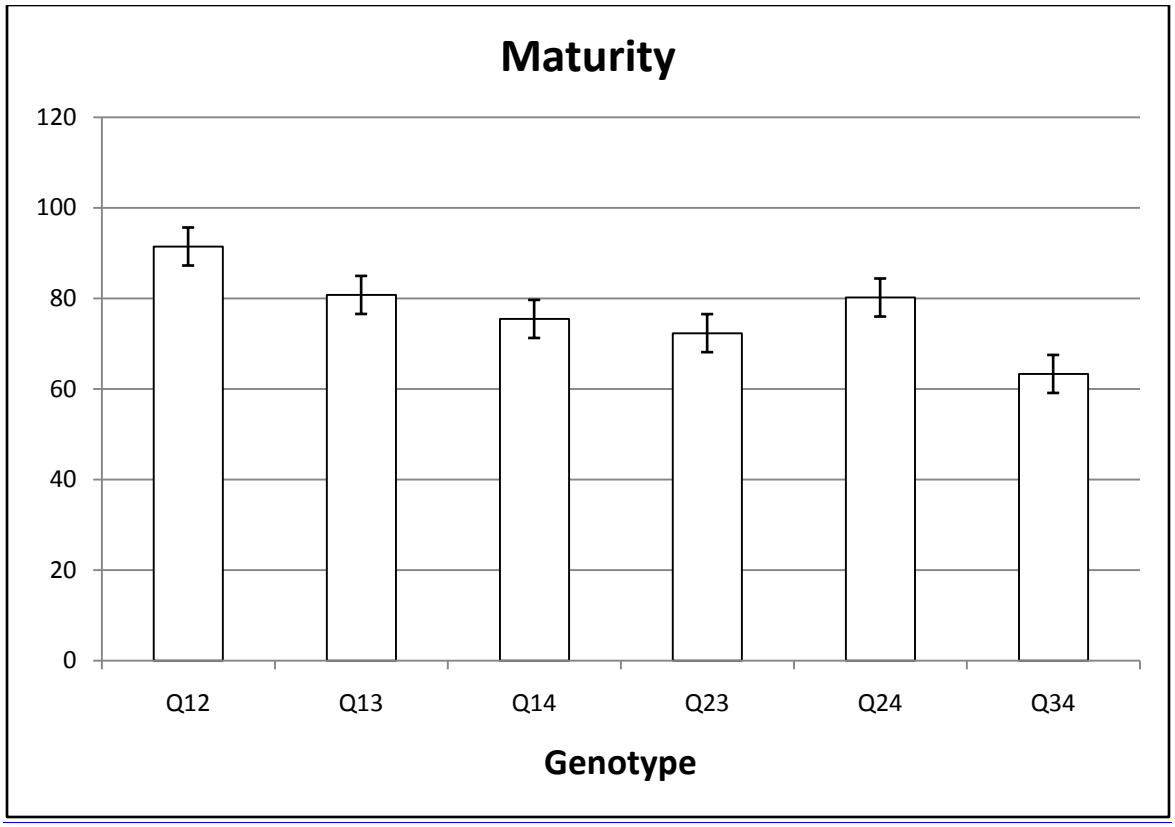

Figure 6. Allelic effects of the QTL for foliage maturity, detected on chromosome III of 'Atlantic' in 2007.

Chapter Three

A microarray approach to identify candidate genes for internal heat necrosis in potato

Per H. McCord, Jason A. Osborne, Bryon R. Sosinski, and G. Craig Yencho

A microarray approach to identify candidate genes for internal heat necrosis in potato

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Abstract

Internal heat necrosis (IHN) is a physiological disorder of potato tubers, resulting in unsightly brown patches of tissue in the parenchyma. Crop losses in the mid-Atlantic and southeastern United States due to IHN are significant, and it is a significant potato production constraint in all regions of the world where significant levels of heat stress are experienced by the potato crop . Using an 11,412 potato gene microarray developed by The Institute for Genome Research (TIGR), we undertook a DNA microarray analysis of tubers grown under normal and high temperature regimes in order to identify genes differentially expressed under heat stress, which could also be associated with IHN. Plants of the IHN-susceptible cultivar 'Atlantic' were raised in growth chambers under normal (20/18°C day/night) or high (28/18°C) temperatures, and tubers were harvested every two weeks from 76 to 118 days after planting (DAP). Total RNA was extracted from tubers and sent to TIGR for hybridization and data generation. Although overall levels of IHN were not significantly different between temperature regimes, levels of IHN were significantly different between the first and last harvest dates under high temperatures, whereas under normal temperatures, levels of IHN remained statistically equivalent. Mixed model analysis of microarray results identified four genes whose expression was significantly different between high and normal temperatures at 76 and 118 DAP. The expression patterns of one gene of unknown function, F3C3.6, were tested via quantitative PCR (qRT-PCR). Between 76 and 118 DAP, the reference or normalizing gene (*EIF*1-α) showed variable expression, violating the assumption for normalizing genes. When *EIF*1-α was not used for normalization, F3C3.6 expression was shown to be unchanged between 76 and 118 DAP under high temperature, and down-regulated between 76 and 118 DAP under normal temperature. Testing of F3C3.6 using field-grown potatoes showed no differences between clean and IHN-affected tubers. Future IHN research should include qRT-PCR of the remaining three candidate genes using both tissue from the original study and field-grown material, and screening of F3C3.6 and any other validated candidate genes for polymorphisms between IHN-resistant and susceptible genotypes.

Introduction

Potatoes are generally adapted to cooler climates. Heat stress in potato induces a number of adverse effects. These include inhibition of tuberization (Ewing 1981), reduced yield (reviewed in Ewing 1981), and reduced tuber dry matter content (Gawronska et al. 1992). Field observations and prior research have indicated that high temperatures can also influence the development of internal heat necrosis, or IHN (Yencho et al. 2008). IHN is a physiological defect of the tuber, characterized by necrotic patches of tissue internal to the vascular ring (Larson and Albert 1945; Henninger et al. 1979). A review of possible methods of control of IHN suggested that breeding for IHN resistance would be the most effective option (Sterrett and Henninger 1997). Henninger et al. (2000) reported that broad-sense heritability for IHN was high, indicating that breeding efforts are indeed warranted. However, screening germplasm for IHN is labor-intensive as there are no external symptoms, so tubers must be dug and sectioned to evaluate IHN levels. Furthermore, expression of IHN symptoms is highly dependent on the environment, requiring multiple years of evaluation in order to make a reliable determination. These obstacles could be mitigated through the use of molecular markers for more rapid and efficient selection.

Gene expression analysis using DNA microarrays can be used to investigate many genes simultaneously, and identify candidate genes for a trait of interest (Salentjin et al. 2003; Lan et al. 2004). These candidates can then be screened for polymorphism. If polymorphism exists, the candidates can potentially serve as molecular markers for breeding if they are associated with the expression of a trait of interest. In addition, candidate genes can serve as a foundation for follow-up studies designed to increase basic knowledge of the biology of the trait of interest. As part of a multi-faceted approach to understand IHN and develop breeding tools for the trait, we conducted a DNA microarray-based expression analysis of potato tubers grown under heat stress during the tuber initiation and bulking phases of tuber development.

We utilized a spotted cDNA microarray developed by The Institute for Genome Research (TIGR, now part of the J. Craig Venter Institute, www.jcvi.org) that contains over 11,400 unique potato genes. This array has been used to identify candidate genes for drought tolerance (Schafleitner et al. 2007) and defense responses to feeding by Colorado potato beetle, *Leptinotarsa declemineata* (Say) (Lawrence et al. 2008). Our primary research objectives were to test the effect of increased temperature on the expression of IHN, and to identify candidate genes involved in the expression of IHN symptoms. Some guidelines for candidate genes for IHN are available. For example, microscopy of IHN-affected tissues has detected disorganization of cells at the periphery of affected areas, thickened, suberized, and irregularly shaped cell walls, a lack of starch, and dark-staining particles in the vacuole (Larson and Albert 1945; Barruzini et al. 1989). Monk et al. (1989) detected the production of free radicals in necrotic tissues, and Davies and Talbot (1989) showed that an IHN-resistant genotype had higher activities of some free-radical scavenging enzymes than a susceptible genotype. In addition to candidate genes for IHN, we expected to identify genes differentially expressed under long-term heat stress, and during tuber development.

Materials and Methods

Plant material, growth chamber, soil mix and fertilizing

The greenhouse and growth chambers used in the study were located at the NC State University Phytotron (www.ncsu.edu/phytotron). 'Atlantic' seed tubers were planted whole in 6 L plastic pots**,** in a Redi-Earth peat-lite substrate (W. R. Grace Co.). Initially, the plants were grown under natural light in one of the Phytotron greenhouses, with 22°C day/18°C night temperatures (15 and 9 hours respectively). However, temperature control was difficult to maintain, and at 46 days after planting (DAP) all plants were moved into two adjacent 'A' growth chambers (both 8.9 m^2) under 16-hour photoperiods, with 20°C day/18°C night temperatures.

Experimental design

The experiment incorporated a factorial design, with temperature and harvest date being the main factors with 2 and 4 levels, respectively (Table 1). This design resulted in eight treatment combinations of time and temperature. The layout in the Phytotron was configured as a completely randomized design (CRD), with the experimental unit consisting of three pots. Four experimental units were randomized to the eight treatment combinations, for a total of 32 observations for each combination. After transfer to the growth chambers, one chamber was programmed to remain at 'normal' temperature (20˚C day/18˚C night) throughout the study. The second chamber was programmed as the 'high' temperature (28˚C day/20˚C night) at 71 DAP, five days before the first harvest. Harvests were conducted at two-week intervals, at 76, 90, 104, and 118 DAP. The final harvest, at 118 DAP, corresponded with the typical age of field plantings during a late harvest of the variety 'Atlantic' in North Carolina.

Harvest and RNA extraction

At each harvest, tubers were counted and weighed in aggregate for each pot. Tuber tissue for RNA extraction was then obtained using a cork borer, and the ends of each sample were trimmed to remove the peel. The tissue was then weighed and placed immediately in liquid nitrogen, and then stored at -80°C until extraction. Equal amounts of tuber tissue (within 0.1 g) were pooled from three plants before RNA extraction. After tissue sampling, all tubers were cut longitudinally and photographed. The photographs were used to score the tubers for presence/absence of IHN. For RNA extraction, frozen tissue was ground with a mortar and pestle in liquid nitrogen, with a small amount of sterilized sand added to aid in grinding. Extractions were performed in 50-mL conical tubes, each using 5 to 10 grams (fresh weight) of ground tuber tissue and 25 mL of extraction buffer. The extraction buffer

was based on a tuber DNA extraction buffer developed by Wulff et al. (2002), with the exception that polyvinylpyrrolidone (PVP) was added to the buffer before autoclaving. The extraction buffer was supplemented with 25 µL β-mercaptoethanol and 12 mL water-saturated phenol. The buffer-phenol mixture was heated to 65°C before the addition of tissue. After tissue was added, tubes were shaken vigorously and incubated at 65°C for 10-15 minutes, with shaking every 2-3 minutes. Following incubation, 12 mL of chloroform: isoamyl alcohol (24:1) was added, and samples were shaken vigorously to form an emulsion. Samples were then centrifuged at 8000 X G and 4°C for 10 minutes. An equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) was then added to the supernatants, shaken, and centrifuged at 8000 X g and 4°C for 8 minutes. Supernatants were then extracted twice with an equal volume of chloroform:isoamyl alcohol, with centrifugation as for the previous step. Following the final organic extraction, 0.5 volumes of 8 M LiCl was mixed with the supernatants, and the samples were incubated overnight at -20°C. RNA was pelleted by centrifugation at 8422 X g and 4°C for 40 minutes, washed with 70% ethanol, dried briefly at 37ºC, and resuspended in150 µL RNase-free water (Sigma-Aldrich, St. Louis, MO). Samples were treated with RNase-free DNase I (New England Biolabs, Ipswich, MA), and then purified and concentrated via chloroform:isoamyl alcohol extraction and alcohol precipitation. RNA extraction from field-grown tubers for quantitative PCR (qRT-PCR) followed essentially the same procedures, but omitted sand in the grinding process, and utilized smaller amounts of tissue and buffer.

Phytotron data analysis

The harvest data from the Phytotron were analyzed using PROC GLM in SAS (SAS, Cary, NC). For tests regarding IHN, only data from tubers actually sampled for RNA extraction were used. Data from all tubers were used when analyzing treatment effects on tuber weight. IHN was scored as present/absent on each sampled tuber. The proportion of affected tubers was transformed using the arcsine function.

Tuber weight was scored as the total weight for the three pots harvested for each replication. IHN incidence (transformed) and tuber weight were each used as dependent variables, with temperature, harvest date, their interaction, and replicates as explanatory variables. Linear contrasts were estimated and tested for all pairwise combinations of temperature, both within and across temperature regimes.

Microarray design

We utilized the microarray developed by TIGR. This is a spotted cDNA array containing 11,412 distinct cDNA clones, spotted in duplicate. The clones were selected from cDNA libraries of a number of potato tissues, including stolon, root, microtuber, dormant tuber, germinating eye, healthy leaf, and *Phytophthora infestans*-challenged leaves, both compatible and incompatible (www.jcvi.org/potato/).

Microarray hybridizations

The microarray experiment was configured as a partially balanced incomplete block (PBIB) design, with the slides considered as blocks. In a PBIB design, not all treatments are present in each block (in this case only two, corresponding to the two fluorescent dyes). It is considered partially balanced because not all pairs of observations occur together the same number of times. For example, 90-day and 118-day harvests grown under normal temperatures were not directly compared (Table 2). This PBIB design does allow for each treatment to be labeled twice with both Cy3 and Cy5, so it is balanced with respect to dye and the number of slides. Total RNA was shipped on dry ice to TIGR, where all samples were processed and hybridizations performed. Upon completion of the hybridizations, we received image and data files from TIGR. These files are available for download and analysis at ftp://ftp.tigr.org/pub/data/s_tuberosum/SGED/116_Yencho/ .

Microarray data processing and analysis

Processing and analysis of the microarray data were performed using SAS version 9 and JMP [Genomics] software versions 6 and 7 (SAS, Cary, NC). The mean spot intensities for each gene were used as the raw data input to the software. The mean spot intensity in this case refers to the average fluorescence across the entire spot. These intensities were averaged over the two spots per gene, and subsequently log-transformed (base 2). Various normalization methods were investigated, including mixed model (Wolfinger et al. 2001), LOESS, or locally weighted scatterplot smoothing (Cleveland 1979), and quantile (Bolstad et al. 2003). Of these methods, LOESS was selected as being the most effective at removing systematic biases from the data. All of these normalization procedures are available in JMP [Genomics].

LOESS normalized data were analyzed gene by gene via PROC MIXED (via the JMP [Genomics] interface) using the following mixed model:

$$\textbf{Log (Intensity)} = \mu + \alpha_i + \beta_j + \delta_{k(I,j)} + \varepsilon_{ijk}$$

Where μ is the mean spot intensity, α_i is the random effect of the *i*th slide, β_j is the fixed effect of the *j*th dye, δ_k is the fixed *k*th treatment effect, and ε_{ijk} is the random error term. T-tests of pairwise comparisons of treatment combinations were performed using standardized least squares means. In addition to testing all pairwise comparisons among treatment combinations, linear contrasts to assess several time by temperature interactions were also estimated. The false discovery rate or FDR (Benjamini and Hochberg 1995) was set at 0.05 to adjust for multiplicity of comparisons. The FDR controls the error rate among tests declared significant at a particular p-value. The chromosomal locations of putative candidate genes were explored using BLAST (Altschul et al. 1990). We utilized the BLASTN algorithm with a word size of 7 and an expect value cutoff of 0.0001, and limited our search to *Solanum* sequences within the NR database.

qRT-PCR analysis

Candidate genes identified from analysis of microarray data were validated by quantitative RT-PCR (qRT-PCR). Primers were designed using Vector NTI Advance, version 10 (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed in 25 µL reaction volumes consisting of 1X RT buffer (Promega, Madison, WI), 6.7 mM dNTPs, 30 units Stop RNase™ inhibitor (5Prime, Hamburg, Germany), 2 µg total RNA, 1 µg oligo-dT primer, and 200 units of M-MLV reverse transcriptase (Promega).
Reactions were incubated for 60 minutes at 42º C. PCR reactions (20 µL) consisted of 1X PCR buffer (10 mM Tris-Cl pH 8.7, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$), 1 µM ROX reference dye (Stratagene, La Jolla, CA), 0.1675X SYBR Green I dye (Stratagene), 0.2 mM (each) dNTPs, 150 nM (each) forward and reverse primers, 1 unit *Taq* polymerase, and 2 µL of diluted (1:10) first-strand cDNA. Two of the candidates, formin homology domain-containing protein (FORMIN) and phosphatase 2C (PHOSPH2C) were assayed using a primer concentration of 300 nM, and SureStart *Taq* polymerase (Stratagene). A Stratagene MX3000P real-time thermocycler was used to carry out qRT-PCR. Reactions (20 µL) were incubated for 3 minutes at 95°C (10 minutes for SureStart *Taq*), followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Fluorescence data was collected throughout the 60°C (annealing) and 72°C (extension) incubations. To generate a dissociation curve, samples were quickly denatured, cooled to 55°C, and slowly heated to 95°C, with fluorescence measurements being collected continuously during the heating phase. Elongation initiation factor 1-α (*EIF*1-α, GenBank accession number AB061263) was used as a reference gene to normalize expression. This gene has been shown to be quite stable in potato across a range of abiotic and biotic stresses (Nicot et al. 2005). No reverse transcriptase controls were included with the initial assays of each sample, and no template controls were included with each assay.

Raw fluorescence data from the extension phase of the PCR was exported to Excel (Microsoft, Redmond, WA). The program LinRegPCR 11.0 (http://LinRegPCR.HFRC.nl) was used to correct for baseline fluorescence, determine efficiency of amplification for each

amplicon, and calculate cycle thresholds (Cts). LinRegPCR uses a two-phase algorithm to reconstruct the log-linear portion of each PCR reaction and determine the baseline correction, which results in the data points from the log-linear phase lying on a straight line. Then, a second algorithm is employed to determine an optimal 'window of linearity' from the log-linear data points for each amplicon. The algorithm chooses this window of data points such that the variation of reaction efficiency amongst members of the same amplicon group is minimized. These data points are then used to calculate the reaction efficiency (which is then averaged across all members of the amplicon group) and Ct for each sample. The algorithms and their results compared to other strategies are described in detail in Ruijter et al. (2009).

The cycle thresholds of the gene of interest and *EIF*1-α, and the efficiencies of each amplicon, were used to estimate the relative change in gene expression between a control or 'calibrator' sample and a treated or 'unknown' sample via efficiency-corrected relative quantification (Pfaffl 2001, Stratagene 2007):

Where:

RQ = quantity relative to calibrator

E(GOI) = efficiency of amplification of gene of interest

E(NOR)= efficiency of amplification of normalizer or housekeeping gene

$\Delta Ct = Ct_{calibrator} - Ct_{unknown}$

Three biological replicates per combination of time and temperature regime were used to validate microarray expression differences. RNA from the original extractions used for the microarray phase of the study was used for cDNA synthesis. In addition, we were interested in comparing our Phytotron-grown samples with field-grown material. Total RNA was therefore extracted from four IHN-affected and four clean tubers, converted to cDNA, and subjected to qRT-PCR. For the microarray samples, the 76-day treatments were considered to be the calibrators within each temperature regime, whereas for the field-grown tubers, the clean (IHN-free) samples were treated as the calibrator. It is important to note that RQ ratios alone are not sufficient to draw conclusions about qRT-PCR results. Some statistical measure of the precision and magnitude of these ratios is also needed. For these

determinations, we utilized the Relative Expression Software Tool (REST) 2008 software package (www.gene-quantification.de/download.html) (Pfaffl et al. 2002). The REST program calculates the RQ ratios, and then applies a randomization test in order to determine standard errors, 95% confidence intervals, and p-values for tests of equality.

Results

Growth chamber studies

Tuber weight was significantly affected by interaction between levels of temperature and time (Table 3, p-value = 0.048). The significance of the interaction is most likely due to the 'crossing' of temperature treatments between 76 and 90 DAP (see Figure 1). There was an increase in tuber weight with successive harvest dates (Figure 1), and tubers grown under the high-temperature regime weighed significantly less than those grown under normal temperatures. There was no main effect for temperature on IHN (Table 3, p-value 0.76), nor was there a significant temperature by time interaction (p-value 0.67). However, some evidence of a time effect on IHN was present (p-value = 0.09, see also Figure 2). When IHN data were viewed as a series of contrasts of pairwise comparisons between harvest dates (Tables 4A, 4B), levels were significantly different between 76 and 118 DAP under high temperatures (4A), whereas under normal temperatures (4B), levels of IHN were not statistically different between any of the harvest dates.

Microarray results

After evaluating expression levels of genes for all pairwise comparisons of treatments, we utilized linear contrasts in PROC MIXED (through JMP [Genomics]) to

compare the change in expression for a given gene between 76 and 118 DAP at high temperatures, to the change in expression of the same gene between 76 and 118 DAP at normal temperatures. Of the 11,412 genes on the array, four genes displayed significantly different changes in expression at FDR = 0.05. These genes and their expression ratios are listed in Table 5. Chromosomal locations were identified for each of these genes via sequence similarity to tomato (*Solanum lycopersicon*), *S. bulbocastanum, S. demissum*, and other *Solanum* species with mapped sequence data. Two of these genes were selected for validation via qRT-PCR: a putative serine threonine kinase (STKIN, GenBank accession number BQ513091), and a gene of unknown function with similarity to a predicted gene, F3C3.6 from *Arabidopsis* (GenBank accession numbers BQ117585 and BQ117586).

Quantitative RT-PCR

The initial primers for STKIN amplified multiple DNA fragments. This gene is located on at least 2 chromosomes (2 and 9), which increases the number of both potential alleles and splice variants. Redesigning the primers did not eliminate multiple fragments. However, one fragment was present in all four IHN-affected field samples, but only 2 of four clean field samples (data not shown). F3C3.6 was successfully tested via qRT-PCR (see Table 6). According to the qRT-PCR data, F3C3.6 was up-regulated at between 76 and 118 DAP under normal temperatures, if *EIF*1-α was used as a normalizing or reference gene. This result is opposite to that derived from the microarray data, which showed a significant down-regulation. However, expression level of *EIF*1-α (as defined by cycle threshold) was significantly different between 118 and 76 DAP under normal temperatures (p-value = 0.004 from a two-sided *t*-test). This violates the assumption that reference genes are not differentially expressed between treatments. When *EIF*1-α expression was not used to normalize the data, F3C3.6 was significantly down-regulated between 76 and 118 DAP at normal temperatures, identical to the results obtained by microarray analysis.

Evidence of differential expression of EIF1-α between 76 and 118 DAP under high temperatures was weak (p-value = 0.13 from a 2-sided *t*-test), but the results of normalization

vs. non-normalization were similar vis-à-vis the microarray analysis. F3C3.6 was shown by qRT-PCR to be up-regulated between 76 and 118 DAP under high temperatures if *EIF*1-α was used for normalization, but showed no significant change in expression (as for the microarray analysis) if *EIF*1-α was not used. With regard to the field-grown tubers, there was no significant difference in expression of F3C3.6 between clean and IHN-affected tubers. There was also no difference in expression of *EIF*1-α (p-value = 0.78).

Discussion

The work described herein was successful in identifying a number of candidate genes differentially expressed in tuber tissue with different levels of IHN. Two of the candidates were also identified in a previous stress-related study utilizing the TIGR microarray. The heat shock protein (HSP, GenBank accession number BQ511262) was shown to be up-regulated under short-term heat stress by Rensink et al. (2005), while it was down-regulated under the long-term heat stress and IHN-promoting conditions of our study. Rensink et al. demonstrated that F3C3.6 was differentially expressed under heat and cold stress, but the direction was not indicated. Heat shock proteins are well known to be involved in response to heat and other stresses where they function as molecular chaperones (reviewed in Wang et al. 2004). The function of F3C3.6 is currently unknown, but its identification in different studies under different stresses (heat and cold, short and long-term) suggests it has an important role to play in the response of potato to temperature stress. The other two candidate genes, STKIN (GenBank accession number BQ513091) and a putative beta-adaptin (B-ADAPT, GenBank accession numbers BQ515087 and BQ515088) are involved in protein regulation and transport, respectively.

STKIN was down-regulated over time under high temperatures, showed no difference in expression over time at normal temperatures, and was expressed at a higher level under IHN –promoting conditions. B-ADAPT showed no difference in expression over time at high temperature, and was up-regulated over time at normal temperature.

When compared across intervals at different temperatures, B-ADAPT showed significant up-regulation under IHN-promoting conditions. These genes have not to our knowledge been identified in any other stress-related studies involving potato. In addition to being identified in our study, HSP is located on chromosome I, where a QTL for IHN has been identified (McCord et al. in preparation). It is encouraging to find reinforcing evidence from separate studies that supports the location of a locus involved in IHN.

There are several follow-up experiments that could be performed to continue this effort to identify candidate genes and markers for IHN. It is probable that more candidate genes exist for IHN than the four we detected. Additional expression analysis studies could be done using more plants per experimental unit. This would give more precise estimates of IHN levels and increase the power to detect differences in IHN between treatments. Genes differentially expressed between these treatments could then be more confidently associated with IHN. Sequencing of candidate genes from IHN-resistant and susceptible individuals could reveal other polymorphisms (SSRs, SNPs, or indels) that could be used to map the candidates. This would be particularly useful when screening our IHN QTL mapping population, as any candidate that maps on or near a QTL for IHN reinforces both the candidacy of the gene, and the veracity of the QTL. Much work remains to be done in order to understand the genetics of internal heat necrosis and develop molecular tools for breeding for IHN resistance, but to our knowledge this is the first functional genomics study of internal heat necrosis.

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Table 1. Experimental variables of the growth chamber experiment using the cultivar 'Atlantic'. The high temperature regime was initiated at 71 DAP, five days before the first harvest.

Temperature Regime	Harvest Date (days after planting)
Normal (20°C day/18°C night), High (28°C day/20°C night)	76, 90, 104, 118

Table 2. Hybridization layout of microarray design. Rep = biological replicate.

Slide	Cy3	Cy5
1	76 Days, Normal, Rep 1	90 Days, Normal, Rep 1
2	90 Days, Normal, Rep 2	104 Days, Normal, Rep 1
3	104 Days, Normal, Rep 2	118 Days, Normal, Rep 1
4	118 Days, Normal, Rep 2	76 Days, High, Rep 1
5	76 Days, High, Rep 2	90 Days, High, Rep 1
6	90 Days, High, Rep 2	104 Days, High, Rep 1
7	104 Days, High, Rep 2	118 Days, High, Rep 1
8	118 Days, High, Rep 2	76 Days, Normal, Rep 2
9	76 Days, Normal, Rep 3	118 Days, Normal, Rep 3
10	118 Days, Normal, Rep 4	118 Days, High, Rep 3
11	118 Days, High, Rep 4	76 Days, High, Rep 3
12	76 Days, High, Rep 4	76 Days, Normal, Rep 4
13	90 Days, Normal, Rep 3	104 Days, High, Rep 3
14	90 Days, High, Rep 4	90 Days, Normal, Rep 4
15	104 Days, High, Rep 4	104 Days, Normal, Rep 3
16	104 Days, Normal, Rep 4	90 Days, High, Rep 4

Table 3. Results of ANOVA of temperature and time (harvest) date on tuber weight (per three pots) and incidence of IHN (arcsine transformed).

Dependent variable	**Source**	**F-value**	**Pr > F**
Tuber weight	Temperature	6.83	0.02
	Time	29.04	<0.0001
	Temperature X Time	3.04	0.048
IHN	Temperature	0.10	0.76
	Time	2.42	0.09
	Temperature X Time	0.52	0.67

Table 4A. P-values of the t-tests of equality of mean IHN levels (arcsine transformed) in potatoes grown under high temperatures.

	76 Days - High	**90 Days- High**	**104 Days- High**
90 Days- High	0.11		
104 Days- High	0.10	0.96	
118 Days- High	0.03	0.49	0.52

Table 4B. P-values of the t-tests of equality of mean IHN levels (arcsine transformed) in potatoes grown under normal temperatures.

	76 Days-Normal	**90 Days-Normal**	**104 Days-Normal**
90 Days-Normal	1.00		
104 Days-Normal	0.73	0.73	
118 Days-Normal	0.14	0.14	0.25

Table 5. List of candidate genes for IHN identified via analysis of microarray data. The first two genes listed were selected for validation by qRT-PCR. [1]More than one accession number indicates sequences derived from the 5' and 3' ends of the original cDNA clone, respectively. [*]QTL for internal heat necrosis have been mapped to this chromosome (McCord et al. in preparation).

GenBank Accession Number(s)[1]	**Gene Name and Symbol**	**Putative Function**	**Chromosome**
BQ117585 BQ117586	Hypothetical protein F3C3.6 (F3C3.6)	Unknown	8
BQ513091	Putative serine/threonine protein kinase (STKIN)	Signal transduction; enzyme regulation	2, 9
BQ511262	Class I heat shock protein (HSP)	Heat shock protein/stress response	1[*], 2, 6
BQ515087 BQ515088	Putative beta-adaptin (B-ADAPT)	Protein transport	8

Table 6. Expression ratios and associated p-values and confidence intervals from microarray and qRT-PCR analyses. STKIN, HSP, and B-ADAPT were not tested via qRT-PCR. H = high temperature; N = normal temperature; Ho = null hypothesis (ratio = 1); NS = not significant at FDR = 0.05. 'IHN' and 'Clean' refer to field-grown samples. Values in parentheses were calculated from non-normalized data (i.e. without taking the expression of EIF1-α into account).

Gene	**Comparison**	**Ratio (array)**	**p-value (Ho)**	**Ratio (qRT-PCR)**	**95% C.I.**	**p-value (Ho)**
F3C3.6	76H vs. 118H	1.09	0.33 NS	0.44 (1.11)	0.35-0.59 (0.59-1.81)	0.00 (0.95)
F3C3.6	76N vs. 118N	2.01	$1.33*10^{-8}$	0.21 (1.92)	0.08-0.47 (1.19-3.51)	0.00 (0.03)
F3C3.6	(76H vs. 118H) vs. (76N vs. 118N)	0.52	$1.68*10^{-5}$			
F3C3.6	Clean vs. IHN			1.12 (1.45)	0.47-2.74 (0.38-4.4)	0.57 (0.40)
STKIN	76H vs. 118H	1.41	$5.92*10^{-5}$			
STKIN	76N vs. 118N	0.78	$2.19*10^{-3}$ NS			
STKIN	(76H vs. 118H) vs. (76N vs. 118N)	1.80	$5.44*10^{-6}$			

Table 6- continued

Gene	Comparison	Ratio (array)	p-value (Ho)	Ratio (qRT-PCR)	95% C.I.	p-value (Ho)
HSP	76H vs. 118H	0.20	$4.51*10^{-9}$			
HSP	76N vs. 118N	0.85	0.34 NS			
HSP	(76H vs. 118H) vs. (76N vs. 118N)	0.24	$6.51*10^{-6}$			
B-ADAPT	76H vs. 118H	0.76	0.003 NS			
B-ADAPT	76N vs. 118N	0.39	$6.47*10^{-11}$			
B-ADAPT	(76H vs. 118H) vs. (76N vs. 118N)	1.97	$6.76*10^{-6}$			

Figure 1. Average tuber weight (per plot) plotted against harvest date. The high temperature regime had a significant negative effect on tuber weight increase.

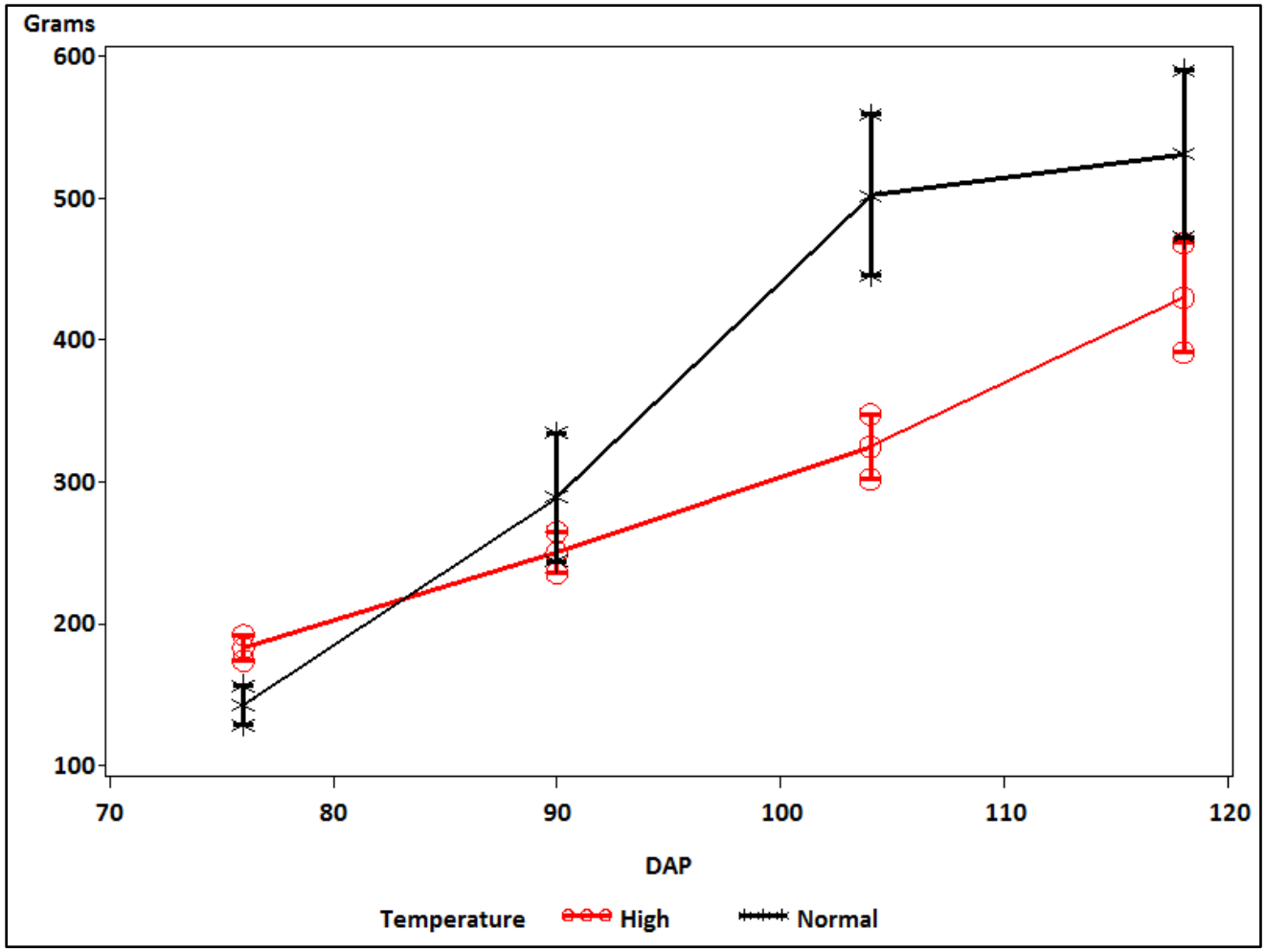
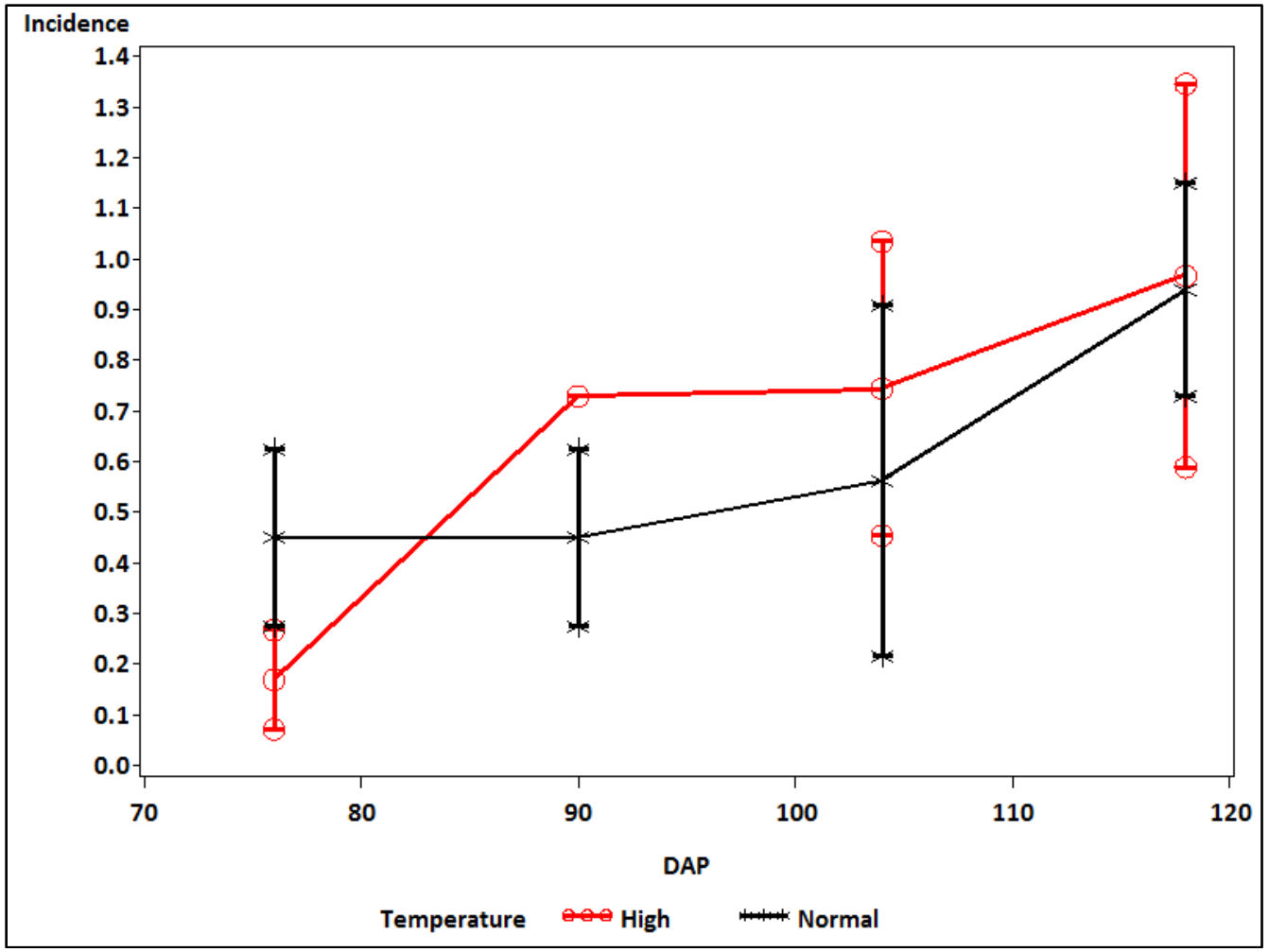

Grams
600
500
400
300
200
100
70
80
90
100
110
120
DAP
Temperature
High
Normal

Figure 2. The progression of IHN symptoms (arcsine transformed) under high and normal temperature regimes. No main effect for temperature on IHN was observed, but the progression of symptoms was different between temperature regimes. Large standard errors inhibited the ability to detect statistically significant differences in IHN levels under normal temperature. Under high temperature, levels of IHN were significantly different between 76 and 118 DAP.


Incidence
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
70
80
90
100
110
120
DAP
Temperature
High
Normal

Chapter Four

The effects of overexpression of a calcium-binding peptide on yield, mineral content, and internal heat necrosis of 'Atlantic' potato

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The effects of overexpression of a calcium-binding peptide on yield, mineral content, and internal heat necrosis of 'Atlantic' potato

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Abstract

Internal heat necrosis, or IHN, is a physiological disorder of potato tubers that may be alleviated by increased tuber calcium. However, establishing a direct connection between calcium and IHN has not been clearly demonstrated. We utilized a transgenic approach to test this association. 'Atlantic' potato was transformed via *Agrobacterium tumefaciens* with a construct containing a maize calreticulin-derived calcium binding peptide (*CBP*), under the control of a CaMV 35S promoter. This peptide has been shown in *Arabidopsis* to increase bioavailable calcium and to increase tolerance to drought and salt stress. To test the effects of the 35S:*CBP* construct on IHN, transgenic and wild-type plants were grown under mild heat stress conditions in a growth chamber. The transgenic lines generally had higher yields than the wild type. However, levels of IHN in transgenic tubers were equal to or higher than wild-type tubers. Quantitative RT-PCR (qRT-PCR) of *CBP* showed no significant differences between transgenic lines, suggesting that differing protein abundance or stability may underlie phenotypic differences between *CBP*-expressing lines. Two native calreticulin genes were also assayed by qRT-PCR to determine if silencing of native genes had occurred, but no evidence was found. Nutrient analysis of calcium and 11 other nutrients was also performed. Calcium levels in leaves of two transgenic lines were significantly higher than wild type 'Atlantic'. Levels of magnesium, manganese, sulfur, and sodium were also higher than wild type Atlantic' in at least one transgenic line. Most of these minerals are involved in photosynthesis; increased amounts in transgenic leaves could be responsible for the increase in tuber yield. In the tubers, mineral levels were not significantly different. It is likely that the expression levels of *CBP* in the tuber are much lower than in the leaves, which would explain the failure to detect any mineral differences. The increase in IHN symptoms could be due to the interruption of a tuber-bound signal from the leaves, or possibly a true effect on tuber minerals that would require larger sample sizes to detect.

Key words: potato, internal heat necrosis, calcium, calcium-binding peptide, yield

Introduction

Calcium is a vital plant nutrient. It is involved in cell division through its effects on the mitotic spindle (reviewed in Inoué 1981) and is an important structural component of the middle lamella (Hopkins 1999). Calcium is necessary for membrane structure and permeability (Hopkins 1999), and serves as a secondary messenger for a wide range of stimuli (reviewed in White and Broadley 2003).

Calcium plays an important role in physiological disorders of crop plants, including bitter pit of apple (Ferguson and Watkins 1989) and blossom end rot of tomato (reviewed in Taylor and Locascio 2004). In potato, calcium supplementation has been shown to mitigate the effects of heat stress on yield (Palta 1996), and to reduce tuber defects such as blackspot bruise (Karlsson et al. 2006) and hollow heart (reviewed in Rex and Mazza 1989). Internal heat necrosis, or IHN, is an important physiological disorder of potato tubers that may be alleviated by increased calcium. IHN is characterized by necrotic patches of tissue in the tuber pith, and can be a major production constraint. However, establishing a clear link between calcium and IHN has been elusive (reviewed in Yencho et al. 2008).

One approach to test the effects of calcium is to use transgenes to modulate intracellular calcium levels. Over-expression of the *Arabidopsis* gene, *AtCAX1*, which encodes a vacuolar H^+/Ca^{2+} antiporter, resulted in increased calcium uptake in potato leaves and tubers (Park et al. 2005a). *AtCAX1* expression has also been shown to increase calcium levels in carrot (Park et al. 2004) and tomato (Park et al. 2005b), where it increased fruit calcium content and shelf life, but was also associated with an increase in blossom end rot. In *Arabidopsis thaliana*, calcium levels were increased 10-fold by heat-shock induction of a transgene encoding an endoplasmic reticulum (ER)-localized calcium-binding peptide (*CBP*) derived from the 3' end of the maize *CRT1* (*Calreticulin1*) gene (Wyatt et al. 2002). In the ER, *CBP* functions as a low-affinity, high-capacity calcium-binding protein. This calcium was shown to be bioavailable by first growing seedlings on normal medium and then transferring them to EGTA-containing medium, where only *CBP* plants continued to grow.

In addition to increasing bioavailable calcium, *CBP* has also been shown to increase tolerance to drought and salt stress in *Arabidopsis* (Lee et al. in preparation).

We elected to transform potato with the same *CBP* used by Wyatt et al. (2002) to answer two important questions. First, will over-expressing *CBP* increase calcium levels in potato, particularly in the tuber? Second, will increasing the concentration of ER calcium in the tuber reduce the incidence of IHN?

Materials and Methods

Vector construct and plant transformation

Agrobacterium tumefaciens (strain GV 3101) containing the plasmid pBIN1301 (Figure 1) was kindly supplied by S.-Y. Lee of the Robertson laboratory. This construct is similar to pBIN2311 used by Wyatt et al. (2002), except that the heat shock promoter was replaced with CaMV35S, and the GFP moiety was removed. Stem sections (without nodes) and leaf segments from virus-free, *in vitro*-cultured 'Atlantic' potato plantlets obtained from the Cornell Uihlein Foundation Seed Potato Farm (Lake Placid, NY), were used as explants. A single colony of *Agrobacterium* was used to inoculate 5 mL of LB broth, which was grown in a shaking incubator for 26 hours at 28ºC. The formulations of all transformation and tissue culture media are listed in Table 1. An aliquot of this culture was diluted 1:10 in LSR medium (slightly modified MS30 medium from Kumar 1995), and explants were incubated in the inoculated medium for 20 minutes in the dark with gentle agitation. Explants were then co-cultivated for three days at 18º C under dim light (16-hour days) on LSR1 medium (also adapted from Kumar 1995) without antibiotics. Thereafter, the explants were transferred to LSR1 medium supplemented with 100 µg/mL kanamycin and 250 µg/mL carbenicillin. Explants were transferred to fresh LSR1 medium (with antibiotics) every 7-10 days. At 21 days post-transformation, callus tissue was visible on both leaf and stem explants. Explants were transferred to regeneration (RG) medium (Johnson et al. 2003) 38 days post-transformation. Explants were transferred to fresh RG medium every 7-11 days.

The first regenerated shoot was observed six weeks post-transformation. When shoots grew large enough to handle, they were excised from the callus tissue and transferred to RIM medium (Beaujean et al. 1998) to stimulate rooting. For a negative control, the pBIN-PLUS vector (van Engelen et al. 1995) without an insert was used. The transformation and regeneration protocol was similar to that used for pBIN1301, with 2 major exceptions: 1. Explants were incubated at 22ºC (vs. 18º C); 2. Timentin was used in place of carbenicillin.

Rooted plantlets were maintained initially in MS basal medium supplemented with appropriate antibiotics. MS basal medium was eventually replaced by MS complete medium, with no noticeable effects. Plants were propagated every 4-6 weeks (on average) to fresh medium to maintain tissue culture stock of all lines. For tuber production, *in vitro* plants were transferred to plug trays filled with Faffard 4P soilless medium (Faffard Inc., Anderson, SC) inside a Humidome™ (American Agritech, Chandler, AZ). The Humidome was placed initially inside a growth chamber. Once established, plants were transferred to 6-inch plastic pots, maintained in the laboratory under shaded natural light for approximately 1 week, and transferred to a greenhouse for first-generation tuber production. Second-generation (vegetative) tubers were also produced in the greenhouse; these tubers were used to plant the replicated trials.

Tests for transgene integration, expression, and silencing

Regenerated lines were initially assayed for the presence of the transgene via PCR. Reactions (20 µL) consisted of 11.76 µL water, 2 µL standard1X PCR buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X 100), 800 µM dNTPs, 300 nM (each) *CBP* forward and reverse primers (Table 2), 1.2 units unit Hot Start *Taq* polymerase (Genesee Scientific), and 2 µL genomic DNA. Thermal cycling conditions were as follows: 15 minutes at 95º C, followed by 30 cycles of 30 seconds at 95º C, 30 seconds at 60º C and 30 seconds at 72º C. Reactions were held for two minutes at 72º C for strand completion, then indefinitely at 4ºC.. PCR products were analyzed via agarose gel electrophoresis using ethidium bromide fluorescence.

Putative transgenic lines were assayed for transgene integration, copy number, and independence via Southern blot. Genomic DNA was digested with *Hin*dIII and separated by overnight electrophoresis on a 1% agarose gel. DNA was transferred to Hybond N+ positively charged nylon membranes (GE Healthcare, Piscataway, NJ) via downward capillary transfer. Membranes were probed with a 152-bp or 350-bp fragment of *CBP* (amplified with *CBP*1 and *CBP* primers, respectively), as well as *nptII*, the selectable marker. Both radiolabeled (^{32}P) and digoxigenin-labeled probes were tested. Radioactive signal was detected using a Kodak (Rochester, NY) phosphor screen and a BioRad (Hercules, CA) FX phosphorimager. Digoxigenin-labeled probes were detected with alkaline phosphatase-conjugated anti-DIG antibodies and CDP substrate, using the DIG Chemiluminescent Detection Kit (Roche, Indianapolis, IN), followed by exposure to X-ray film.

Lines were also tested for *CBP* expression and possible native *CRT* gene silencing via quantitative RT-PCR (qRT-PCR). For total RNA extraction, tissue from *in vitro*-grown plantlets was frozen in liquid nitrogen and ground in a 1.7 mL microcentrifuge tube using a plastic pestle. Preheated (65ºC) 2X CTAB extraction buffer (www.cipotato.org/csd/materials/Molecular/Molecular1.pdf) with an approximately equal volume of water-saturated phenol was added to the ground tissue, and samples were incubated in a water bath at 65ºC for 10-15 minutes, with frequent shaking. After addition of 0.5 volumes of 24:1 chloroform:isoamyl alcohol, tubes were shaken well to mix and centrifuged for 8 minutes at 13000 rpm at 4ºC. Supernatants were extracted once more with chloroform:isoamyl alcohol. Lithium chloride was added to a final concentration of ~4 M, and samples were incubated overnight at -20ºC. RNA was pelleted by centrifugation for 20 minutes at 13000 rpm at 4ºC, washed with 70 % ethanol, dried briefly, and resuspended in RNase-free water. The concentration and purity of RNA samples was assayed with a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). RNA samples were incubated with RNase-free DNase I (New England Biolabs, Ipswich, MA) at 37ºC for 10 minutes, and DNAse was removed by extraction with 24:1 chloroform:isoamyl alcohol.

First strand cDNA synthesis was performed in a reaction volume of 25 µL consisting of 1X RT buffer (Promega, Madison, WI), 6.7 mM dNTPs, 30 units Stop RNase™ inhibitor (5 Prime, Hamburg Germany), 1 µg total RNA, 500 ng oligo-dT primer, and 200 units of M-MLV reverse transcriptase (Promega). Reactions were incubated for 60 minutes at 42ºC.

All lines were assayed using qRT-PCR (SYBR Green I method) for the expression of 35S:*CBP* and two native potato genes, *StCRT-2* and *StCRT-3* (GenBank accession numbers DV627409 and CO502133). These genes have significant similarity to *Arabidopsis* genes *AtCRT-2* (GenBank accession number NM100791) and *AtCRT-3* (GenBank accession number NM100718), respectively. Elongation initiation factor 1-α (*EIF*1-α, GenBank accession number AB061263) was also assayed as a normalizing (housekeeping) control (Nicot et al. 2005). The GenBank accession number, primer sequences, and expected product size are listed for each gene in Table 2. Quantitative PCR was performed using a Stratagene MX3000P QRT-PCR System (Stratagene, La Jolla, CA). PCR reactions (20 µL) consisted of 1X PCR buffer (10 mM Tris-Cl pH 8.7, 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$), 1 µM ROX reference dye, 0.1675X SYBR Green I dye, 0.2 mM (each) dNTPs, 150 nM (each) forward and reverse primers, 1 unit Hot Start *Taq* polymerase (Genesee Scientific, San Diego, CA), and 1 µL first-strand cDNA. Reactions were incubated for 15 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Fluorescence data was collected throughout the 60°C (annealing) and 72°C (extension) incubations. To generate a dissociation curve, samples were quickly denatured, cooled to 55°C, and slowly heated to 95°C, with fluorescence measurements being collected continuously. No-reverse transcriptase controls using *CBP* primers were included with the initial assays of each sample, and no-template controls generally using *EIF*1-α primers were included with each assay. For fluorescence baseline correction, amplicon efficiency estimation, and cycle threshold (Ct) calculations, raw fluorescence data from the extension phase (72°C) was exported into Excel (Microsoft, Redmond, WA) for use with LinRegPCR 11.0 (http://LinRegPCR.HFRC.nl).

The Ct and efficiency estimates were then used to derive efficiency-corrected expression ratios of gene expression (Pfaffl, 2001) and associated statistics using REST 2008 (www.gene-quantification.de/download.html).

Replicated growth chamber trial

Second-generation (vegetative) tubers of all lines were planted in 10-inch plastic pots containing Faffard 2P (Faffard Inc., Anderson, SC) soilless medium. Plants were maintained in two 'A' growth chambers (9 m^2) at the NCSU Phytotron. With respect to IHN, the experiment was configured as a randomized complete block design (RCBD) with 2 replications (chambers), and 5 pots per line per chamber. For statistical analyses of tuber yield and tuber/leaf calcium, the experiment was treated as a completely randomized design (CRD) with 1 plant per plot and 5 replications per line per chamber. Initially, the chambers were maintained at 22ºC/18ºC with a 14-hour daylength. Then, to simulate the warming late spring and summer growing conditions, temperatures were increased at 33 days after planting (DAP) to 26.7ºC/ 21.1ºC; daylength remained constant (14 hours). Since Faffard 2P is a nutrient-fortified medium, plants were irrigated with distilled water only, except for the period from 46 to 57 DAP, when they were irrigated with the standard Phytotron nutrient solution (http://www.ncsu.edu/phytotron/manual.pdf). All potatoes were harvested at 91 DAP, with potatoes from individual plants placed into separate bags. The following day, all potatoes from each bag were weighed in aggregate. To score levels of IHN, the 20 largest tubers from the 5 plants of each line (per chamber) were quartered longitudinally, and the mean severity rating from 9-1 (Sterrett and Henninger 1997) as well as percentage of incidence were calculated based on the appearance of the most affected quarters from each potato sampled. In cases where 20 tubers were not available, all tubers were quartered and rated. Data for all phenotypic measures were analyzed via SAS version 8.2 (SAS, Cary, NC) using PROC GLM.

Comparisons between treatment means of all transgenic lines (including the negative control) and 'Atlantic' were performed using Dunnett's two-sided t-test (comparison-wise $\alpha = 0.05$).

Mineral analysis

Leaf tissue was sampled from all plants in the second chamber prior to harvest and stored in plastic bags at -80ºC. In the case of tubers, a single tuber from each pot (except for the negative control, which had very small tubers) in the second chamber was peeled, weighed, placed in a plastic bag, and stored at -20ºC. Due to the cost of mineral analyses, tissue was sampled from only one chamber. Both leaf and tuber tissue samples were lyophilized prior to analysis. Paired leaf and tuber samples from five plants per line were submitted to A&L Eastern Laboratories (Richmond, VA) for analysis of calcium and other mineral content. In addition to calcium, leaf and tuber samples were analyzed for levels of phosphorus, potassium, sulfur, magnesium, sodium, iron, aluminum, manganese, copper, zinc, and boron. Data were analyzed using SAS (SAS Cary, NC) routines PROC CORR and PROC GLM. In cases where GLM detected significant differences ($p < 0.05$), Dunnett's procedure was used to compare means of transgenic lines to 'Atlantic'.

Results

CBP integration

All putative transgenic lines appeared to have integrated the transgene when assayed by PCR (Figure 2). At this point, plants had been maintained on Basal Medium without antibiotics for over a year with no sign of *Agrobacterium* contamination, and testing via PCR for contamination yielded negative results (data not shown). Southern blot analyses (not shown) indicated that several transgenic lines were the result of the same transgenic event. Based on information from the Southern blots, phenotypic data, and tissue culture history, lines *CBP*-1, *CBP*-5, *CBP*-8, *CBP*-9, and *CBP*-12 were determined to be independent.

These lines, plus wild-type ‘Atlantic’ and the pBIN-PLUS negative control, were those chosen for the growth chamber, mineral and qRT-PCR analyses.

Growth chamber study

Yield was greater for 3 of the 5 transgenic lines when compared to ‘Atlantic’ (Figure 3). The line with the highest yield, *CBP*-8, yielded 51.9% more than ‘Atlantic’ on a per-plant basis. This is in contrast to the results obtained by Park et al. (2005a), who showed no significant difference in yield between their wild-type line (Russet Norkotah) and sCAX1-transformed lines. The pBIN-PLUS negative control plants were much slower to sprout than all other lines, and so were less mature at harvest, with correspondingly lower yields. There was a significant chamber effect (p-value 0.02), with plants in the second chamber producing an average of 182.7 g per pot compared with 203.1 g per pot in the first chamber. There was no significant clone by chamber interaction (p-value 0.64).

Levels of IHN (as measured by percent incidence) were higher in absolute terms for 4 of 5 transgenic lines, although only one of these lines showed greater incidence when measured in terms of statistical significance (Figure 4). Compared to yield, which had 10 replications across the two chambers, the IHN measurements were only replicated twice, resulting in lower statistical power. Line *CBP*-8, the line with highest yield, also had the highest levels of IHN incidence (70%). The negative control plots (pBIN-PLUS) had zero incidence of IHN. However, the pBIN-PLUS plants were slower to emerge than the other lines, and their tubers were not as mature at harvest (see Figure 3). Since younger tubers are less likely to express IHN symptoms (P. McCord, personal observation), the negative control results are not considered reliable.

Calcium and other minerals

Leaf calcium levels were significantly higher than ‘Atlantic’ in *CBP* lines 1 and 8; none of the transgenic lines (including the vector control) had significantly less leaf calcium than ‘Atlantic’ (Table 3).

In leaves, levels of magnesium, manganese, sodium, and sulfur were significantly higher than 'Atlantic' in at least one of the transgenic lines. None of the lines were significantly lower that 'Atlantic' for any of the other nutrients besides calcium. In tubers, there were no significant differences in mineral content between any of the lines (data not shown).

Quantitative RT-PCR

As expected, there was no expression of *CBP* in either wild-type 'Atlantic' or pBINPLUS. All transgenic lines expressed *CBP* (Table 4). To compare expression of *CBP* amongst transformed lines, we arbitrarily chose *CBP*-5, the line with the lowest levels of IHN, as the 'control' for purposes of testing. Line *CBP*-12 expressed higher levels of *CBP* compared to *CBP*-5 (Table 5A); all other transgenic lines were equivalent. All lines ('Atlantic' and transgenics) expressed *StCRT-3* at an equivalent level. The expression levels of *StCRT-2* were highly variable within lines (see standard errors in Table 5B), resulting in no significant differences between lines.

Discussion

As mentioned in the introduction, expression of *CBP* in *Arabidopsis* has been shown to increase bioavailable calcium under calcium-deficient conditions (Wyatt et al. 2002). We did not impose a calcium deficiency in our study, but we have also demonstrated an increase in leaf calcium through expression of *CBP* in potato. As in our case, *CBP* expression has additional effects on the elemental composition of *Arabidopsis.* Transgenic *Arabidopsis* plants have been shown to accumulate higher levels of potassium and lower levels of sodium than wild-type plants (Lee et al., in prep). In comparison, the leaves of transgenic 'Atlantic' potato plants did not accumulate extra potassium, and had higher levels of magnesium, manganese, sulfur, and sodium. The C-terminal portion of calreticulin, from which *CBP* was derived, is known to bind magnesium as well as calcium (Baksh and Michalak 1991). However, *CBP* expression does not increase magnesium levels in *Arabidopsis*

(Lee et al. in preparation), although it does increase the concentration of chlorophyll in leaf tissue. The significance of increased Mg in potato, but not Arabidopsis, is not clear. There may be compensatory mechanisms for equalizing charges across membranes that involve different transporters in the two species. It is also possible that the magnesium-binding ability of *CBP* is the cause of increased magnesium in leaves of transgenic potato plants. Magnesium is a cofactor for many reactions, including the ATPase activity of the chaperone, BiP, which is an abundant protein in the ER (Haas 1994).

Expression of *CBP* in *Arabidopsis* increases seed weight (Lee et al., in preparation), and in potato, has a definite enhancing effect on tuber yield (Figure 3). Obviously, *Arabidopsis* seeds and potato tubers are very different organs, but both are sink tissues, so these effects may have a common root cause related to *CBP*. It is unclear what causes the increase in yield, but it may have to do with increased photosynthesis. Magnesium, manganese, and sulfur are all involved in the reactions of photosynthesis (Hopkins 1999). It is possible that increased levels of these minerals in transgenic plants increased their rate or efficiency of photosynthesis, which translated into increased tuber yield. An increase in yield was a serendipitous result of this study, and certainly warrants further research; replicated field trials would be particularly useful in this regard.

Our tests of gene silencing of *CBP* and native calreticulins, and measures of leaf calcium, were precipitated by preliminary results. Before the growth chamber study was completed, an initial analysis of tuber calcium (via inductively coupled plasma emission spectrometry) was undertaken using peeled tuber tissue from greenhouse-grown plants. Lines were not replicated, but an overall trend was apparent; all transgenic lines had less tuber calcium than both wild-type and negative control lines (data not shown). This apparent decrease in tuber calcium, combined with failure to decrease levels of IHN in the Phytotron study, led to the development of two testable hypotheses: 1. Over-expression of *CBP* increased bioavailable calcium, which was then preferentially translocated to the leaves. This caused a calcium deficiency in the tubers, causing an increase in IHN.

2. Expressing *CBP* under the control of CaMV35S led to its over-expression and silencing; native calreticulin genes, which do share some sequence similarity with *CBP* (data not shown), were also unintentionally silenced. This led to decreased calcium binding ability, lower calcium levels, and increasing IHN. To test the first hypothesis, we measured total calcium in both leaf and tuber tissues from the replicated Phytotron study. Calcium was significantly higher in leaves for two transgenic lines, but there was no significant difference between tubers. It appears that an increase in leaf calcium does not come at the expense of tuber calcium. It is unclear why there was no difference in mineral status between wild-type and transgenic tubers, but it may also have to do with varying expression levels of the 35S-driven construct. CaMV 35S is considered to be a constitutive promoter, but there are reports of tissue-specific differences in expression levels of genes under its control (Williamson et al. 1989; Sunilkumar et al. 2002; Samac et al. 2004). We used leaf tissue for our qRT-PCR analyses, but it would be wise to test *CBP* expression in tuber tissue as well. Although *CBP* was more abundant in leaves of one line (*CBP*-12), this line did not display significantly different levels of IHN as compared to 'Atlantic'. Rather than levels of mRNA, the differences between lines are most likely due to variable protein expression. A *CBP*-specific antibody is available (D. Robertson, personal communication), and in the future, Western blots to assay *CBP* protein levels will be performed.

We tested the second hypothesis via qRT-PCR of *CBP* and 2 native calreticulin genes. *CBP* was expressed in all transgenic lines, and there was no detectable variation in leaf expression. The second native calreticulin, *StCRT-3*, showed stable and equivalent expression across all lines, i.e. there was no evidence of silencing. There was also no evidence of silencing of *StCRT-2*, though this gene did show significant variability within lines, suggesting it is highly sensitive to differences in environmental conditions. Based on these results, we believe we can eliminate gene silencing as contributing to our observations of IHN and nutrient levels. However, as for the first hypothesis, this does not rule out tissue-specific expression of the construct.

What, then, could be the cause of at least one transgenic line experiencing levels of IHN incidence, which are higher than wild-type? First, we reiterate that more replications would increase the statistical power of the analyses. A study with three or four replications would greatly increase our confidence in the effects of *CBP* on IHN. But these tests are very costly to conduct and would require considerably more growth chamber space. There were statistically significant differences between leaves of wild-type 'Atlantic' and one or more transgenic lines for calcium, manganese, magnesium, sodium, and sulfur. These lines were in almost all cases *CBP*-8 and/or *CBP*-1, which also had the highest absolute levels of IHN. It is possible that over-expression of *CBP* in the leaves interrupts signaling from the leaves to the tubers, which is involved in expression of IHN. This hypothesis could be addressed in a straightforward manner by grafting transgenic scions onto wild-type rootstocks, and vice versa. Another possibility is that *CBP* is indeed expressed in the tubers and interrupts their nutrient status, but at levels difficult to detect without more replicates.

We were able to successfully demonstrate an increase in leaf calcium through over-expression of *CBP*. Expression in tubers may require the use of a tuber-specific promoter (such as patatin or granule bound starch synthase), but *CBP* is active in potato. A clear link between calcium and internal heat necrosis remains elusive. Future research should continue to look at calcium, but should also include manganese, magnesium, sodium, and sulfur, since levels of these minerals were also different in transgenic potatoes with increased levels of IHN.

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Table 1. Formulations of transformation and tissue culture media used in the study**.** All quantities are per liter**.** Solutions were adjusted to pH 5.7 with 1M KOH after addition of MS salts and sucrose**.** Vitamins, hormones, and antibiotics were added after autoclaving. Timentin was used instead of carbenicillin for transformation of plants with the empty pBIN-PLUS vector.

	LSR	**LSR1**	**RG**	**RIM**	**Basal Medium**
MS complete medium					4.4g
MS basal salts	4.4 g	4.4 g	4.3 g	4.4 g	
Sucrose	30 g	30 g	30 g	30 g	30 g
Phytagel (Sigma-Aldrich, St. Louis, MO)		3 g	3 g	3 g	3 g
Thiamine			900 µg		
Gibberelic acid (GA3)		20 µg	2 mg		
Indole-acetic acid (IAA)				100 µg	
Naphthalene-acetic acid (NAA)		200 µg			
Zeatin riboside		2 mg	808 µg		
Carbenicillin		250 mg	250 mg	250 mg	250 mg
Kanamycin		100 mg	100 mg	100 mg	100 mg
Timentin		200 mg	200 mg	200 mg	200 mg

Table 2. GenBank accession numbers, primer sequences, and expected product sizes of genes assayed by ***q***RT-PCR**.** Primer sequences for *CBP* and *EIF*1-α were originally published in Wyatt et al. (2002) and Nicot et al. (2005), respectively**.**

Name	**GenBank Accession**	**Forward primer (5'-3')**	**Reverse Primer (5'-3')**	**Expected Size (base pairs)**
CBP	AF190454	ACATGCATGCCCCTATGATTG ACA ACC	ACATGCATGCCGATCTAGAGC TCGTC	377
*CBP*1	AF190454	GCCGAGAAAAAGAAGGAAGA AGAG	GCTCGTCGTGCTTCTCATCATC	152
*EIF*1-*α*	AB061263	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	101
StCRT-2	DV627409	CTGGTAGCTTGTACTCTGACT GGGA	TTCTTGGCTTCGGGATCAGTTAT	172
StCRT-3	CO502133	CAGACTGGGATATCTTTCCTC CTCG	CCATCCTCTTCATCATCCCAGTG	187

Table 3. Leaf tissue nutrient composition of 'Atlantic', pBIN-PLUS, and *CBP*-transformed lines. Clones are listed in order of their IHN incidence rankings (highest at top). Numbers in each cell refer to ranks; ranks with an asterisk are significantly different from 'Atlantic' according to Dunnett's 2-tailed t-test ($\alpha = 0.05$). [1]Significant using a 1-tailed Dunnett's t-test (treatment > control).

Clone	Al	B	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
***CBP*-8**	2	3	1*	6	7	7	2[1]	1*	2[1]	6	1*	6
***CBP*-1**	4	1	2*	5	6	4	1*	2*	1*	2	2*	3
***CBP*-12**	5	4	3	2	4	5	3	3*	4	7	6	5
***CBP*-9**	1	2	4	1	2	3	5	4	3	1	7	1
Atlantic	6	7	5	4	1	2	4	5	5	3	3	4
***CBP*-5**	7	6	6	4	3	1	6	6	6	4	5	4
pBINPLUS	3	5	7	3	5	6	7	7	7	5	4	2

Table 4. Mean cycle threshold (Ct) from qRT-PCR analysis of *CBP* expression in 'Atlantic', pBIN-PLUS, and *CBP*-transformed lines. Each line was assayed three times (biological replicates). [1]No Ct was detected for one replication of both 'Atlantic' and pBIN-PLUS.

Line	**Ct**
'Atlantic'	38.17[1]
pBIN-PLUS	37.40[1]
CBP-1	26.85
CBP-5	27.33
CBP-8	26.79
CBP-9	27.70
CBP-12	25.44

Table 5A. Relative expression ratios of *CBP* in transgenic lines. Each line was compared to *CBP*-5, the line with the lowest absolute levels of IHN.

Line	**Expression Ratio**	**95% C.I.**	**p-value**
CBP-1	0.99	0.74 - 1.21	0.94
CBP-8	0.97	0.59 - 1.37	0.81
CBP-9	0.95	0.63 - 1.26	0.95
CBP-12	2.31	1.48 – 4.36	0.02

Table 5B. Relative expression ratios of *StCRT*-2 and *StCRT*-3 in transgenic lines. Expression ratios are relative to 'Atlantic'.

Line	**Gene**	**Expression Ratio**	**Standard Error**	**95% C.I.**	**p-value**
pBIN-PLUS	*StCRT*-2	3.10	1.44 - 6.60	1.22 - 11.60	0.06
CBP-1	*StCRT*-2	0.93	0.56 - 1.60	0.39 - 2.18	0.63
CBP-5	*StCRT*-2	4.14	1.05 - 14.12	0.64 - 24.81	0.20
CBP-8	*StCRT*-2	1.54	0.36 - 4.39	0.22 - 7.14	0.63
CBP-9	*StCRT*-2	2.52	0.40 - 14.26	0.24 - 25.07	0.45
CBP-12	*StCRT*-2	2.77	1.27 - 6.04	0.77 - 7.77	0.10
pBIN-PLUS	*StCRT*-3	1.26	0.96 - 1.73	0.86 - 2.00	0.325
CBP-1	*StCRT*-3	1.41	0.93 - 2.01	0.78 - 2.98	0.43
CBP-5	*StCRT*-3	1.22	0.75 - 1.91	0.55 - 2.60	0.41
CBP-8	*StCRT*-3	0.92	0.69 - 1.31	0.68 - 1.34	0.65
CBP-9	*StCRT*-3	1.64	1.05 - 2.43	0.86 - 3.61	0.12
CBP-12	*StCRT*-3	1.38	0.98 - 2.16	0.74 - 2.43	0.20

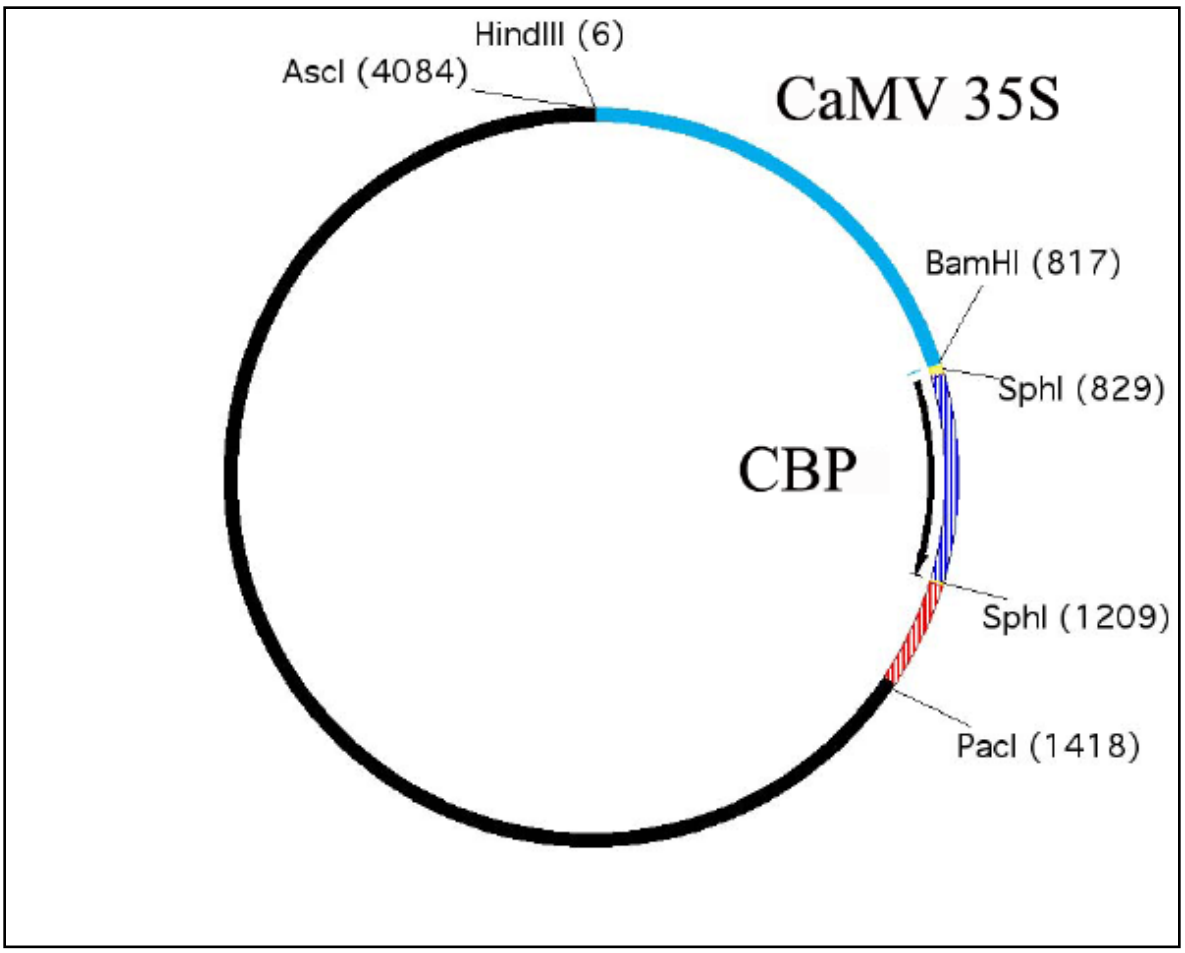


Figure 1. Vector construct pBIN1301. Figure courtesy of S-Y. Lee and D. Robertson.

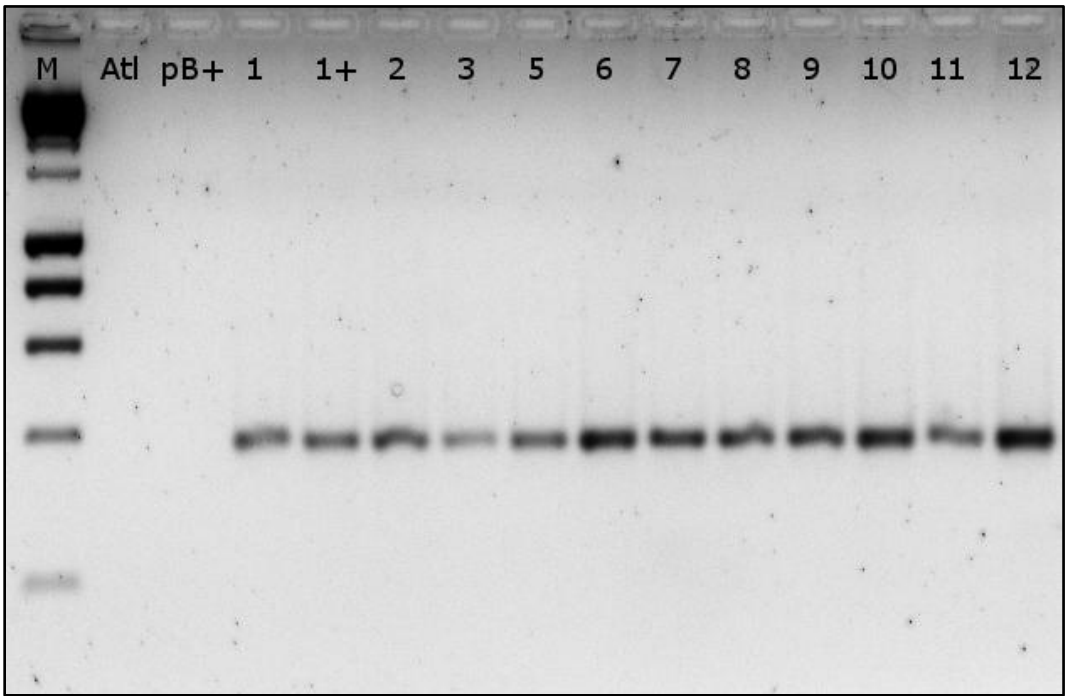


Figure 2. PCR analysis of transgenic lines for *CBP* integration. M = size standard; Atl = 'Atlantic' (wild-type); pB+ = pBIN-PLUS (negative control).

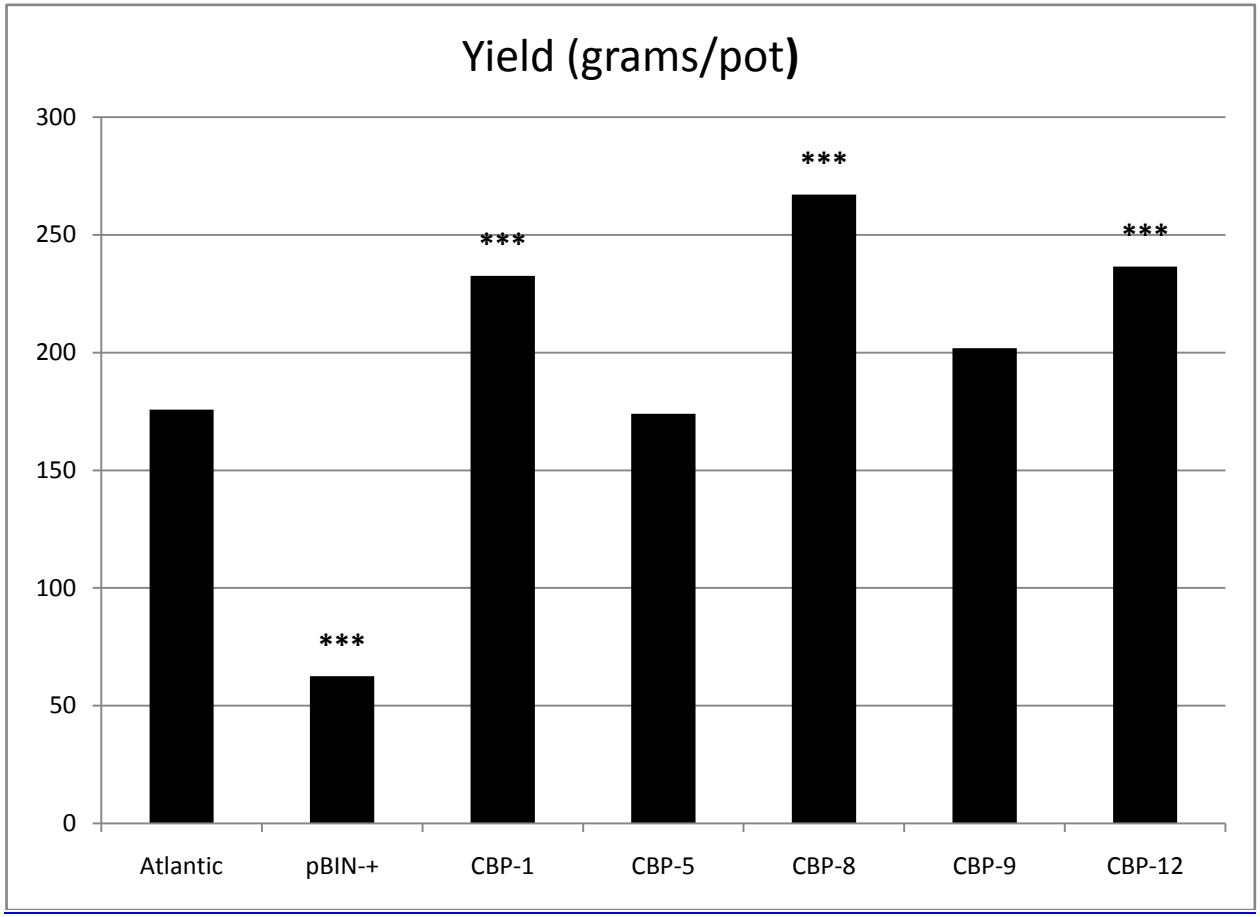
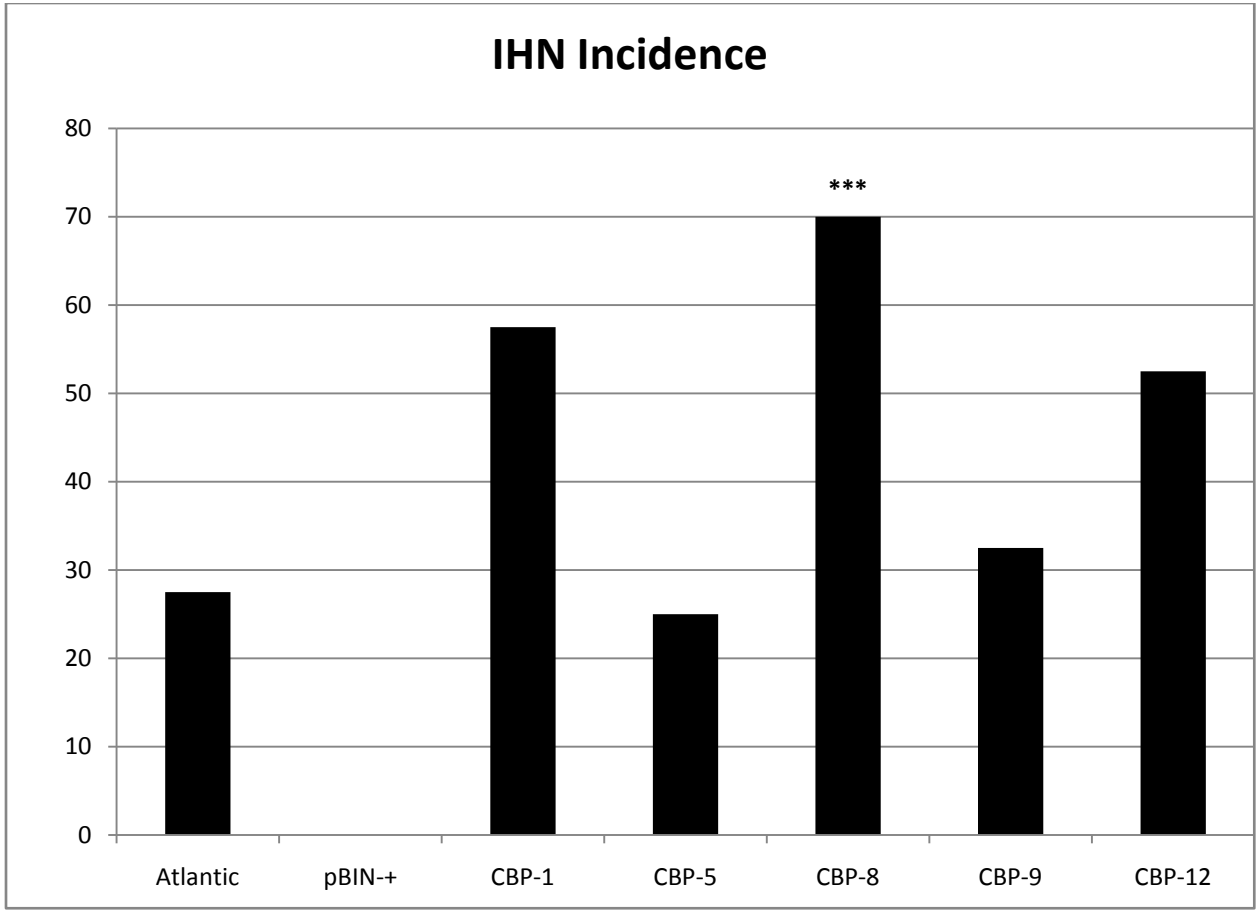


Figure 3. Tuber yield (in grams per pot) of all lines tested in the Phytotron. Bars labeled with asterisks are significantly different from 'Atlantic' according to Dunnett's procedure ($\alpha = .05$).

Figure 4. Percent incidence of IHN across all lines. Bars labeled with asterisks are significantly different from 'Atlantic' according to Dunnett's procedure ($\alpha = 0.05$).


IHN Incidence
80
70
60
50
40
30
20
10
0
***
Atlantic
pBIN-+
CBP-1
CBP-5
CBP-8
CBP-9
CBP-12

SUMMARY

Internal heat necrosis (IHN) is a serious physiological disorder of potato tubers, characterized by brown patches of tissue internal to the vascular ring. Despite its significant economic impact, particularly in the mid-Atlantic United States, relatively little progress has been made towards understanding and ameliorating IHN. An older review (Sterrett and Henninger 1997) of methods of control for IHN recommended control through superior genetics, i.e. breeding IHN-resistant cultivars. The obstacles to breeding IHN-resistant cultivars include the quantitative nature of the trait, and the time and effort required to screen germplasm. Because IHN resistance/susceptibility is quantitative, it is difficult to predict the performance of progeny in a given cross. There are no external or above-ground symptoms of IHN, so potatoes must be dug and cut to observe symptoms. Also, certain environmental conditions unfavorable to potato growth must be present in order for IHN symptoms to manifest the*mse*lves reliably. In order to understand the genetics of the disorder, and generate useful information for breeders, I undertook genetic, genomic and transgenic research approaches to improve understanding of IHN.

The first and largest project, described in Chapter 1, was to generate a linkage map from a population of tetraploid potato segregating for IHN resistance, and identify quantitative trait loci (QTL) for the trait. The goal was not to clone or even finely map QTL for IHN, but to obtain a general estimate of the number, location, and effects of loci governing the trait. This was the 'genetic' component of my research. My initial plan was to develop linkage maps and identify QTL in two distinct populations of moderate size, rather than a single, larger population. This would sacrifice some precision in estimating location and effects of QTL, but would allow me to observe whether a common set of loci govern the trait, or if QTL for IHN are population-specific. Due to time constraints (my other two projects), I forsook developing a complete map of the second population, and decided to use that population to test QTL located in the first population.

Generation and scoring of AFLP and SSR markers was relatively straightforward; development of the linkage map was much more challenging. Mapping in species with polysomic inheritance requires many more markers to cover the genome than in a diploid organism. Despite generating over 650 AFLP and SSR markers, I was not able to identify all 48 chromosomes. However, map coverage was sufficient to perform interval mapping of QTL on many chromosomes, and testing of single markers was performed in cases where this was not possible. I was successful in identifying QTL for increased resistance on a number of chromosomes, primarily from 'Atlantic', the susceptible parent of the primary mapping population. Several QTL were also found on B1829-5, the resistant parent. It was particularly encouraging to see many of these QTL map to the same or similar locations over several years of research. Most QTL displayed dominant gene action, and were associated with resistance. This is the likely explanation for the phenotypic distribution of IHN, which is highly skewed towards resistance.

In addition to mapping QTL in B2721, a small number of markers from the initial mapping population were tested in the second population; time constraints did not permit the testing of all significant markers. Of the 12 AFLP markers tested, 7 appeared to be polymorphic in the second population, and one (E38M58_135) was significantly associated with reduced susceptibility to IHN. In addition, the SSR marker STI032, which was linked to IHN susceptibility in B2721, was closely linked (2.9 cM) in repulsion to a NC206-specific marker that was also associated with susceptibility. This demonstrates that at least two regions associated with IHN are not unique to the original mapping population, but more research is needed to confirm whether or not the markers that were not significant represent loci unique to the primary population. Of course, more markers need to be tested in the secondary population, and I hope that this research takes place; it will be very interesting to see which markers and/or QTL are population-specific, and which are not.

The mapping population also segregated for other agronomic traits, and I collected data and searched for QTL for these traits as well. I noticed in my trips to the field that the population segregated for foliage maturity and for flower color. The results of QTL mapping for tuber dry matter, specific gravity, foliage maturity, skin texture, yield, and flower color are presented in Chapter 2. By mapping these traits, I found QTL for foliage maturity that support the results of previous research, and located a major QTL for flower color on the same chromosome as the *R* locus for red anthocyanin production (van Eck et al. 1993). I also detected QTL across multiple years for yield, specific gravity, dry matter, and skin texture. To my knowledge, skin texture has not been previously mapped.

My second project, described in Chapter 3, encompassed the genomic aspect of my research. Our research group was successful in having our cDNA microarray proposal, titled "Understanding the genetics of internal heat necrosis (IHN) in potato (*Solanum tuberosum*) via microarray analysis of gene expression", funded by The Institute for Genome Research (TIGR, now part of the J. Craig Venter Institute, Rockville, MD). This experience gave me confidence in grant writing, which will likely be an important part of my career. The initial proposal was to analyze the gene expression of IHN-resistant and IHN-susceptible full-sib genotypes from our primary mapping population, under normal and IHN-inducing conditions. However, the lack of sufficient tuber stock of IHN-resistant and IHN-susceptible clones to conduct these experiments restricted our experiment to testing only 'Atlantic', which is IHN-susceptible.

Some very important lessons were learned from this project. Perhaps foremost was that the performance of plants in growth chambers can be very different from the field, or even the greenhouse; the plants and tubers looked quite different from field-grown material. Second, the combination of heat stress and tuber development causes thousands of genes to be differentially expressed. Microarray experiments are powerful, but the amount of data they generate can be daunting. Third, we were not able to detect a significant main effect for temperature with respect to internal heat necrosis.

This was probably due to a combination of the atypical growing conditions in the growth chambers, and the relatively small number of samples we could screen due to space constraints in the growth chambers, and cost limits on the number of samples that could be analyzed on the arrays. Despite these challenges, there were detectable differences in the progression of IHN symptoms over time between temperature treatments, and with some excellent advice from Dr. Jason Osborne, I was able to appropriately analyze the microarray data for genes differentially expressed between treatments that resulted in differences in IHN. This process reduced the number of differentially expressed genes from over 3,000 to just four candidates. Validation of one of these candidate genes by quantitative RT-PCR added another tool to my academic toolbox. In addition to the valuable lessons learned during the various stages of the 'genomics' project, I identified a number of candidate genes for IHN. Of particular interest to me is the fact that the validated gene, F3C3.6, has an unknown function. One of the candidate genes was located on chromosome I, where I was able to map a QTL associated with IHN. This is a good example of the value of a multi-faceted research approach to address a scientific question. Not only did I gain a wider variety of skills and exposure to different techniques, but I saw firsthand how different projects could produce complementary results. The next steps in utilizing the genomics data generated should include the screening of candidate genes for polymorphism (ideally SNPs), the mapping of these SNPs to compare their location with QTL for IHN, additional qRT-PCR to validate the remaining candidate genes, and qRT-PCR of IHN-free and IHN-affected tubers grown in actual field conditions.

The transgenic approach, described in Chapter 4, was the third component of my research. Whereas the genetic and genomic projects had been global in scope, the 'TransAtlantic' project (named for the cultivar that was transformed) sought to address a specific question. Calcium has been linked to IHN in some studies, but not in others (see Yencho et al. 2008 for a review of IHN).

The availability of a calcium-binding peptide derived from maize calreticulin, which had been shown to increase both bioavailable calcium and stress tolerance in *Arabidopsis* (Wyatt et al. 2002; Lee et al. 2009, in preparation), provided an opportunity to test the calcium hypothesis directly by attempting to increase calcium in 'Atlantic' potato tubers, and observing the effect on IHN.

As with the microarray study, space constraints did not allow for a great deal of replication, but we were able to detect some statistically significant results. First, the calcium-binding peptide or *CBP* increased tuber yield, in one case by approximately 50% over wild-type 'Atlantic'. This was completely unexpected. Also unexpected was the fact that *CBP* did not reduce IHN, but in at least one transgenic line, made it worse. These results (with respect to IHN) made some sense, as preliminary tissue calcium data suggested that transgenic lines had lower levels of tuber calcium and higher levels of leaf calcium compared to wild-type 'Atlantic'. Two possible explanations for these preliminary results are as follows. First, overexpression of *CBP* via the 35S promoter lead to silencing of *CBP* and native calreticulins, reducing the plants' ability to retain calcium. Alternatively, increased mobility of calcium lead to its translocation to the leaves, reducing tuber calcium and increasing heat necrosis. Expression analysis of *CBP* and two different native calreticulin genes showed *CBP* expression in all transgenic lines, and no evidence of silencing. Replicated analyses of leaf and tuber calcium confirmed that some transgenic lines had higher leaf calcium, but showed no difference in tuber calcium. Eleven other minerals were also analyzed; several of these (magnesium, manganese, sulfur, and sodium) were higher in leaves of transgenic lines, but were not different in tubers. This could mean that nutrient status of the leaves affects internal heat necrosis, or that important nutrient differences in the tubers are subtle enough to require larger sample sizes for detection. The fact that several other nutrients besides calcium were affected by the transgene suggests that other nutrients could be involved in IHN.

Although the link between calcium and IHN remains elusive, or perhaps is not as important as suggested by other studies (Tzeng et al. 1986; Clough 1994), this project was not unsuccessful. We were able to increase calcium in certain parts of the potato plant, and we were able to influence IHN albeit not in the desired direction. Furthermore, *CBP* appears to have an enhancing effect on yield, which is very interesting from a breeder's point of view. Quantitative RT-PCR of tuber RNA and Western blots of leaf and tuber tissue still need to be performed in order to determine protein levels and spatiality of *CBP* expression. It may well be that *CBP* will still be useful in testing the calcium hypothesis, perhaps under a tuber-specific promoter. We plan to re-examine the effects of our 35S:*CBP* construct on yield and IHN via a larger-scale replicated field trial.

At the completion of this significant amount of work, I think it is wise and useful to ask myself two questions: 1. Would you do anything differently? 2. Was it worth it to do three unique projects? The answer to the first question is 'Of course.' By focusing on one large population for QTL mapping, I believe our map would have had better coverage, and we would likely have detected more QTL, particularly those with smaller effects. We would also have obtained more accurate estimates of QTL effects, both by virtue of the larger population size (i.e. more phenotypic data points), and from better marker coverage (more genotypic data points).

With respect to the 'TransAtlantic' project, we had access to a tuber-specific promoter fairly early in the course of the project. I think it would have been a more targeted and elegant experiment to switch promoters and direct *CBP* expression to the tubers *per se*. In general, I found that sometimes I got away with doing things 'the easy way', but when I didn't, I lost valuable time. Learning to slow down, plan thoroughly, and 'do it right the first time' is something I've gotten better at, but I still need to improve. The answer to the second question is a resounding YES! My research was broad, and it was a challenge to change gears from one project to another in quick succession, often on the same day.

However, the multi-faceted approach was successful in providing me with a broad array of skills, ideas, and experiences. It was also refreshing to switch from one project to another if one became particularly frustrating. Overall, I feel I was successful, both in contributing to the knowledge of internal heat necrosis, and in gaining the skills, experience, and personal acquaintances to help me be a successful plant scientist.

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