Development of Molecular Markers for Marker-Assisted Selection of Dieback Disease Resistance in Lettuce (*Lactuca sativa*)

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**Abstract**

Lettuce dieback disease is widespread in commercially grown romaine and leaf-type lettuces, but not in iceberg-type cultivars. The disease is caused by two closely related *Tombusviruses*: *Tomato bushy stunt virus* (TBSV) and *Lettuce necrotic stunt virus* (LNSV). A single dominant gene (*Tvrl*) on chromosomal linkage group 2 has been found to be responsible for the resistance observed in modern iceberg lettuces. A population of 192 F₈ recombinant inbred lines (RILs) developed from crosses between the susceptible romaine cultivar ‘Valmaine’ and the resistant iceberg cultivars ‘Salinas 88’ and ‘Salinas’ was used to pinpoint the position of the resistance gene with EST-based molecular markers. Nine markers closely linked to the *Tvrl* gene were subsequently tested for association with resistance in a set of 68 accessions from all horticultural types of lettuce. Sequencing of the marker that matched precisely with resistance revealed the presence of three haplotypes in *L. sativa*, two of them associated with resistance. High-resolution DNA melting analysis, which allows detection of all three haplotypes in a single analysis, was successfully applied to study the marker-trait association. Results confirmed the usefulness of the marker for marker-assisted selection in all types of cultivated lettuce.

**INTRODUCTION**

Cultivated lettuce (*Lactuca sativa* L.) is the world’s most important leafy salad vegetable and is especially valuable as a commercial crop in Asia, North and Central America, and Europe (Lebeda et al., 2007). In the United States, California is the largest lettuce producing state with an estimated 72% of the total U.S. production of crisphead lettuce and 79% of combined romaine, leaf, and butterhead lettuce (CLGRP, 2009). Arizona is the second largest lettuce-producing state, annually accounting for 18-20% of the total lettuce production in the United States (Ryder, 1997). Since the early 1990s a previously uncharacterized dieback disease of lettuce has been observed throughout the main producing areas of California and Arizona. Symptoms of the disease include mottling, yellowing, and necrosis of older leaves, and stunting and death of plants (Fig. 1) (Wisler and Duffus, 2000; Obermeier et al., 2001; Grube et al., 2005). The disease affects romaine and leaf type lettuce often leading to 60% or more crop loss (Wisler and Duffus, 2000). An attempt to isolate a causal agent yielded two closely related soilborne viruses from the family *Tombusviridae* - *Tomato bushy stunt virus* (TBSV) and *Lettuce necrotic stunt virus* (LNSV) (Obermeier et al., 2001). These viruses have no known vector and they seem to be transmitted through infested soil and water (Wisler and Duffus, 2000). Available evidence suggests that in the mid-1920s the same or a similar *Tombusvirus* caused widespread destruction of the crisphead cultivar ‘New York’ that was the predominant cultivar planted throughout California at the time. The disease, which was referred to as brown blight, disappeared when the resistant crisphead cultivar ‘Imperial’ replaced the former crisphead type (Jagger, 1940; Wisler and Duffus, 2000). Similar to
brown blight disease, dieback disease was never observed in any modern crisphead cultivar (mostly referred to as iceberg type) that has cv. ‘Imperial’ in the pedigree (Grube et al., 2005). Existing data indicate that resistance to dieback in modern iceberg cultivars is still effective despite having had regular exposure to the pathogen for several decades (Grube et al., 2005).

Tvr1 is the single dominant gene responsible for dieback resistance in iceberg cv. ‘Salinas’. Molecular marker analysis has allowed for the positioning of this gene on chromosomal linkage group 2 (Grube et al., 2005). The same genomic area contains a dieback resistant allele that originates from the primitive romaine-like accession ‘PI491224’. Analysis of offspring from a cross between cv. ‘Salinas’ and ‘PI491224’ has indicated that the two resistance genes are either allelic or closely linked (Grube et al., 2005).

The objectives of the present study were to 1. fine-map the location of Tvr1 from cv. ‘Salinas’ with closely linked molecular markers, 2. test for association between molecular markers linked to Tvr1 and resistance observed on a set of diverse accessions, and 3. develop molecular markers to enable marker-assisted selection in all horticultural types of lettuce.

MATERIALS AND METHODS

Linkage Mapping Population and Association Mapping Set

Recombinant-inbred lines (RILs) were derived from a cross between a F1 of cv. ‘Valmaine’ (dieback susceptible romaine type) × cv. ‘Salinas 88’ and cv. ‘Salinas’ (dieback resistant iceberg type). One hundred and ninety two F8 RILs were screened for resistance to dieback in multiple trials and genotyped with molecular markers.

A set of 68 accessions consisting of 8 Batavia types, 5 butterhead types, 5 iceberg types, 5 Latin types, 9 leaf types, 31 romaine types, and 5 stem types (Table 1) was used in association mapping. For each horticultural type both dieback resistant and susceptible accessions were selected, with the exception of iceberg lettuce, where only resistant cultivars were available, and the Latin type, where only susceptible cultivars were available.

Assessment of Dieback Resistance

Dieback resistance data were obtained from field observations as previously described (Simko and Hu, 2008). Susceptibility was evaluated by seeding lettuce directly in the field in Salinas, CA, from which LNSV had previously been isolated from plants exhibiting characteristic dieback symptoms (Grube et al., 2005). Plants were evaluated weekly for disease symptoms, and the percentage of plants that showed typical dieback symptoms (or were dead due to dieback) was recorded at harvest maturity. Accessions with <5% symptomatic plants were considered to be resistant. All accessions were tested in at least three independent field trials and classified into one of the two groups.

DNA Isolation, PCR, Allele Detection, Product Sequencing, and Phylogenetic Analysis

Genomic DNA was extracted from lyophilized leaf tissue using the NucleoSpin Plant II kit (Macherey-Nagel, Bethlehem, PA). Primer pairs were designed for each marker from EST (expressed sequence tag) sequence (CGPDB 2009) with the PRIMER 3 software (Rozen and Skaletsky 2000). PCR was performed in a 20 µl volume containing 10 ng of genomic DNA as a template, 200 µmol/L of each dNTP, 1X Standard Taq PCR buffer with 1.5 mmol/L MgCl₂, 1.2 U Taq polymerase (all from New England Biolabs, Ipswich, MA), and forward and reverse primers at a concentration of 0.25 µmol/L each. The reaction conditions were as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 57°C (or 61°C) for 30 s, and 72°C for 30 s, with a final extension of 72°C for 5 min. PCR products were separated either by gel electrophoresis on a combination agarose and Synergel (Diversified Biotech, Boston, MA) gel, or capillary electrophoresis using a
HDA-GT12 DNA analyzer with Bioculator software (eGene, Irvine, CA).

For sequencing, PCR products were treated with Exonuclease I and Antarctic Phosphatase (both from New England Biolabs, Ipswich, MA). DNA sequencing was performed using ABI BigDye Terminator (v3.1; Applied Biosystems, Foster City, CA) on an ABI 3730xl DNA sequencing machine. DNA sequences were analyzed with CodonCode Aligner v. 2.0.6 (CodonCode Corporation, Dedham, MA) and tagged with Haploview v. 4.2 (Barrett et al., 2005). Phylogenetic analysis on sequences was conducted with the neighbor-joining algorithm using the computer software Clustal X v. 1.81 (Thompson et al., 1997).

High-Resolution DNA Melting Curve (HRM) Analysis

Alternatively, EST-derived markers were screened for polymorphism using high-resolution melting curve analysis. PCR was performed in 10 µl reactions containing 10 ng DNA, 200 µmol/L of each dNTP, 0.6 U Taq polymerase, 1X Standard Taq buffer with 1.5 mmol/L MgCl₂ (all from New England Biolab, Ipswich, MA), 1X LC Green Plus Melting Dye (Idaho Technology, Salt Lake City, UT), 0.25 µmol/L of each primer, and 15 µ of mineral oil (USB Corporation, Cleveland, OH). PCR cycling conditions were as follows: initial denaturation of 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, 61-64°C (depending on the specific primer combination) for 30 s, and 72°C for 30 s, with a final extension of 72°C for 5 min; after which samples were heated to 95°C for 30 s and cooled to 25°C to facilitate heteroduplex formation. Melting-curve analysis was performed on a LightScanner System (Idaho Technology).

Linkage Mapping

One hundred and ninety two RILs derived from a cross between a F₁ of cv. ‘Valmaine’ (dieback susceptible romaine type) × cv. ‘Salinas 88’ and cv. ‘Salinas’ (dieback resistant iceberg type) were genotyped with EST-derived markers selected from a microarray-based study carried out on the ‘Salinas’ × ‘UC96US23’ (Lactuca serriola) population (CGPDB 2009). Marker polymorphism was tested with HRM analysis, gel or capillary electrophoresis, or sequencing. Statistical analysis was performed by MapManager QTX software (Manly et al., 2001).

Association Mapping and Assessment of Population Structure

Association mapping was performed on a set of 68 accessions from seven horticultural types of lettuce (Table 1). Markers closely linked to the Tvr1 gene as identified by linkage mapping were sequenced for each accession, analyzed for polymorphism with CodonCode, SNPs were tagged in Haploview, and analyzed for association with TASSEL v. 2.0.1 (Bradbury et al., 2007). Prior to association analysis, the population structure in the set of 68 accessions was assessed with thirty EST-SSR markers (with polymorphism $U_\text{He} \geq 0.28$) distributed throughout the genome (Simko 2009) using the computer program STRUCTURE 2.2 (Falush et al., 2007).

RESULTS AND DISCUSSION

Of the 192 RILs genotyped and phenotyped in multiple field trials, 92 were resistant and 100 were susceptible to the disease, which is consistent with 1:1 ratio of segregation and a single gene effect. Linkage mapping identified nine markers that were closely linked to the Tvr1 gene ($p<0.001$). The largest phenotypic effect ($R^2=100\%$) was observed for markers Cntg4252 and Cntg10192 that co-segregated with the resistance allele in the (‘Valmaine’ × ‘Salinas 88’) × ‘Salinas’ mapping population (Fig. 2). In the consensus chromosomal linkage map, which also includes the segregation observed in the ‘Salinas’ × ‘UC96US23’ population, the two markers are positioned 0.5 cM apart. Sequencing of nine markers from the Tvr1 region revealed 160 SNPs, of which 60 were non-redundant for discrimination of haplotypes. Eighteen of these SNPs were significantly ($p<0.001$) associated with the resistance allele. Significant SNPs were detected on all markers with the exception of marker Cntg4252, for which the best value
was \( p=0.0042 \). The SNPs with the largest effect (\( R^2=100\% \)) were found on marker Cntg10192 at positions 54 and 72. Both of these SNPs have C \( \Rightarrow \) T base substitutions where T is associated with resistance and C with susceptibility to dieback; however, both mutations are synonymous and do not lead to changes in amino acids. The third SNP in the same marker is located at position 100 and explains about 41\% (\( p<0.001 \)) of the trait variation. In comparison, none of the SNPs observed in marker Cntg4252 were significantly associated with resistance, though the marker co-segregated with the resistance allele in the linkage mapping population. It is not uncommon for markers closely linked with a trait in a mapping population to not show association when tested on a set of diverse accessions. This was well documented in lettuce (Moreno-Vázquez et al., 2003), as well as in other crops (Lawson et al., 1998), where markers most tightly linked to a resistance gene were not useful for diagnostic purposes when tested in a large collection of cultivars. Therefore, an important requirement for any molecular marker used in MAS is not just its applicability in a specific cross, but its association in a wide and diverse gene pool.

To further investigate polymorphism in marker Cntg10192, five accessions from three wild lettuce species that are sexually compatible with \( L. \) sativa were sequenced. This set consisted of two accessions from each of \( L. \) serriola (‘UC96US23’, ‘PI274808’) and \( L. \) virosa (‘IVT280’, ‘PI274378d’), and one accession from \( L. \) saligna (‘PI490999’). All of the accessions are resistant to lettuce dieback, with the exception of \( L. \) serriola accession ‘PI274808’. Sequencing revealed eight additional SNPs in marker Cntg10192 (GenBank numbers GQ341336 to GQ341438). The combination of SNPs discriminated six haplotypes: \( L. \) virosa, \( L. \) saligna, \( L. \) serriola, two \( L. \) sativa, and a haplotype that includes accessions from both \( L. \) sativa and \( L. \) serriola (Fig. 3). The two \( L. \) sativa haplotypes appear to be associated with different resistance alleles at \( Tvr1 \) – the ones present in cv. ‘Salinas’ and ‘PI491224’, respectively. \( L. \) virosa and \( L. \) saligna haplotypes show less similarity to \( L. \) sativa than those of \( L. \) serriola haplotypes. This observation corresponds to decreasing sexual compatibility of the two species with \( L. \) sativa (Lebeda et al., 2007) and also with transferability of EST-SSR markers between species (Simko, 2009). Five of the identified haplotypes contained accessions resistant to dieback, while a sole haplotype contained only accessions susceptible to the disease. The susceptible haplotype can be separated from resistant haplotypes by a single SNP at position 72. While all resistant haplotypes have the T allele at this locus, the susceptible haplotype bears the C allele.

In order to identify \( L. \) sativa haplotypes in Cntg10192 without sequencing, we developed a primer pair that allowed for detection of polymorphism through high-resolution melting analysis. These primers amplify a 185 bp product that contains three SNPs detected in \( L. \) sativa (and also \( L. \) serriola) at positions 54, 72, and 100. The approach worked well and we were able to discriminate among four haplotypes of \( L. \) sativa and \( L. \) serriola in a single assay. Application of high-resolution DNA melting analysis is now part of our breeding program. Marker-assisted selection allows us to substantially reduce field-based testing for disease resistance. Instead of several years of replicated trials, a single DNA analysis detects resistant genotypes. Moreover, we can identify and select homozygous individuals whose offspring do not segregate for resistance in the following generation.

**ACKNOWLEDGEMENTS**

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**Literature Cited**


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Table 1. List of 68 accessions used in association mapping.

<table>
<thead>
<tr>
<th>Resistant</th>
<th>Susceptible</th>
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<tr>
<td>01-778M, 01-781M, 01-789M, Balady Banha, Balady Barrage, Bandit, Bibb, Blonde Lente à Monter, Celtuce, Cobham Green, Cracoviensis, Defender, Drumhead White Cabbage, Express, Grand Rapids, Great Lakes 54, La Brillante, Margarita, Pacific, PI491214, Ruby Ruffles, Salad Bowl, Salinas, Sea Green, Sharp Shooter, Skyway, Sx08-003, Sx08-004, Sx08-005, Sx08-006, Sx08-007, Sx08-008, Tania, Triple Threat, Two Star, Vanguard</td>
<td>Balady Aswan, Barnwood Gem, Batavia Reine des Glaces, Clemente, Darkland, Gallega, Gladiator, Gorilla, Green Forest, Green Towers, Heart's Delight, Holborn's Standard, Iceberg, King Henry, Little Gem, Lobjoits Cos, Lolla Rossa, MayKing, Merlot, New York, North Star, Paris White, Parris Island Cos, Pavane, PI207490, Reuben's Red, Short Guzmaine, Sucrine, Tall Guzmaine, Valmaine, White Paris, Xena</td>
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Fig. 1. Dieback symptoms on romaine-type lettuce. Plants infected in early stages of growth are severely stunted (top); those infected in later stages show severe mottling and yellowing of leaves (bottom). Photographs were taken 11 weeks after planting.
Fig. 2. Part of chromosomal linkage group 2 with nine markers linked to the \textit{Tvr1} gene. Results from linkage (left) and association mapping (right) indicate percentage of phenotypic variation in the disease resistance (R²%) explained by the marker. All markers in both analyses were significantly correlated ($p<0.001$) with resistance, with the exception of marker Cntg4252 in association mapping that was significant at $p<0.05$. Association mapping results show the effect of the most significant SNP in each marker. Note the different scales for marker effect.

Fig. 3. Haplotypes detected in the marker Cntg10192. Similarity among haplotypes is illustrated on the left, while position of SNPs, reaction to virus infection, and number of accessions in each haplotype is shown at right.