Construction of High-Density Linkage Maps and Identification of Quantitative Trait Loci Associated with Verticillium Wilt Resistance in Autotetraploid Alfalfa (*Medicago sativa* L.)

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Abstract

Verticillium wilt (VW) of alfalfa is a devastating disease that causes forage yield reductions of up to 50% in the northern United States and Canada. The most effective method for controlling the disease is through the development and use of resistant varieties. To identify quantitative trait loci (QTL) for VW resistance in alfalfa, we used a full-sib population segregating for VW resistance. High-density linkage maps for both resistant and susceptible parents were constructed using single-dose alleles of single-nucleotide polymorphism markers generated by genotyping-by-sequencing. Five QTL associated with VW resistance were identified and they were in four linkage groups (4D, 6B, 6D, and 8C). Of those, three QTL (*qVW-6D-1*, *qVW-6D-2*, and *qVW-8C*) had higher logarithm

of odds. Two putative candidates of nucleotide-binding site leucine-rich repeat disease resistance genes were identified in the QTL intervals of *qVW-6D-2* and *qVW-8C*, respectively. The result agreed with our previous studies, in which similar resistance loci were identified in an association panel using genome-wide association. The results provide insight into the quantitative resistance to VW in alfalfa. The resistance loci and closely linked markers identified in the present study can be used in developing new alfalfa varieties with enhanced resistance to VW.

Keywords: genotyping-by-sequencing, linkage map, QTL mapping, single-dose allele, Verticillium wilt

Verticillium wilt (VW) is a devastating disease in alfalfa and causes serious forage yield loss in the northern United States and Canada (Viands et al. 1992). For instance, the annual loss in hay production caused by VW in the state of Wyoming was estimated to be \$1.7 million per year (Page et al. 1992). VW of alfalfa is a soilborne disease caused by the fungal pathogen *Verticillium alfalfae* (Inderbitzin et al. 2011). The fungus is seed transmitted, which results in phytosanitary concerns for the movement of potentially infected seed lots (Christen and Peaden 1982). The disease symptoms initiate with leaf tip chlorosis, leaf desiccation, and abscission and, as the disease progresses, infected plants eventually wilt and die (Leath and Pennypacker 1990). Methods such as eradication of broadleaf weed hosts and crop rotation may be used for controlling the disease (Huang 2003; Leath and Pennypacker 1990). However, due to other dispersal factors, including insects, manure, wind, and water, the control strategies may not be effective. Furthermore, the fungus can be seed transmitted, resulting in introduction of the pathogen into uninfested areas (Huang 2003). The most effective method for controlling the disease is to develop and use resistant varieties (Leath and Pennypacker 1990; Peaden et al. 1985). It has been suggested that

alfalfa cultivars require at least 60% resistant plants for reasonable protection against the disease (Grau 1991). It has been reported that the yearly benefit of using highly resistant varieties ranged from \$21 to \$44/ha (Smith et al. 1995).

Genetics of plant resistance to VW has been investigated in alfalfa varieties. Viands (1985) compared the inheritance of VW resistance in alfalfa plants selected from the European cultivars, Maris Kabul and Vertus, and suggested that a major dominant gene may control VW resistance in Maris Kabul, while more complex additive effects were found in Vertus. Conventional selection for VW resistance in alfalfa requires evaluating host response to the pathogen and is time consuming (Grau 1991). Developing molecular markers associated with VW resistance and the use of marker-assisted selection (MAS) would greatly accelerate the breeding program. A molecular marker has been reported for detecting the pathogen, and a quantitative PCR assay has been developed for quantifying *V. alfalfae* DNA in alfalfa (Larsen et al. 2007). Inderbitzin et al. (2013) developed a PCR assay using a set of primers from the ribosomal internal transcribed spacer region from the pathogen that gives diagnostic amplicons for *V. alfalfae*. However, the assays can only detect the presence of the pathogen in alfalfa plants but do not show whether the host plant is resistant to the disease. Developing a molecular marker associated with host plant resistance would provide a robust method for selecting resistant alfalfa varieties.

Alfalfa is an autotetraploid species with $2n = 4X = 32$. Alfalfa plants outcross and, consequently, are highly heterozygous (Julier et al. 2003), which makes it a considerable challenge to genotype individuals. However, recent advances in next-generation sequencing provide a new strategy to generate cost-effective, high-density, genome-wide single-nucleotide polymorphisms (SNPs) (Elshire et al. 2011). We previously used genotyping-by-sequencing (GBS) and genome-wide association studies (GWAS) to identify loci associated with VW resistance in a panel of breeding lines in alfalfa (Yu et al. 2016). We identified 10 SNP markers associated with VW resistance. Sequence alignment to the *M. truncatula* genome revealed multiple resistance loci, several of which are linked to known resistance genes.

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Quantitative traits such as biotic and abiotic stress resistance are most likely under the control of multiple genes and interact with environmental factors. Previous reports suggested that VW resistance in alfalfa is likely controlled quantitatively by multiple genes (Yu et al. 2016). In the present study, to further investigate the genetic base of VW resistance in alfalfa, we used a full-sib population segregated for resistance or susceptibility to the disease for mapping quantitative trait loci (QTL) associated with VW resistance in alfalfa.

Materials and Methods

Plant materials and phenotyping. An alfalfa population containing 188 F1 progeny was developed from a cross between two parental plants of 55V50-118 (susceptible, P1) $\times$ 55V50-58 (resistant, P2) at S & W Seeds Company (Nampa, ID, U.S.A.). The two parents and the F1 progeny were propagated by stem cuttings to make clones. The cloned plants were transplanted into a pot containing Pro-Mix potting soil (Sun-Gro, Denver, CO, U.S.A.) when new roots were developed (about 4 weeks after cutting). Both parents and the progeny were placed in a randomized complete block design with three replications. They were then evaluated for resistance to VW using the standard assay, as previously described by Zhang et al. (2014).

The disease severity scores of three replicated clones from each individual were analyzed using analysis of variance (ANOVA) (Table 1). Distribution of VW resistance was performed on the obtained disease severity scores of the population using JMP software (SAS Online Doc 9.3; SAS Institute Inc., Cary, NC, U.S.A.) and subjected to QTL mapping. A nonparametric Kruskal-Wallis test was also used for analyzing the disease scores and a Dunn post hoc test was performed with a *P* value adjusted by the Benjamini-Hochberg method (Benjamini and Hochberg 1995) using the statistical software R.

GBS and SNP calling. High molecular weight DNA was extracted from leaves of the original plants used for making the clones using the Qiagen DNeasy 96 Plant kit, following the manufacturer's protocol (Qiagen). GBS was carried out as described by Elshire et al. (2011). Briefly, DNA samples from individuals of the mapping population were digested by ApeKI restriction enzyme. GBS libraries were prepared by ligating the digested DNA to unique nucleotide adapters (barcodes) in 96-plex, followed by PCR amplification. Sequencing was performed on the Illumina HiSeq2000 instrument (Illumina, San Diego, CA, U.S.A.), using two lanes.

The Universal Network Enabled Analysis Kit (UNEAK) pipeline was used for genotype calling according to Lu et al. (2013). Briefly, sequence reads were trimmed to 64 bases. A minimum of five were used for the tag number count step using the UQseqToTagCountPlugin command line build into the UNEAK. An error tolerance of 0.03 for tag pair alignment was used to assign genotypes. A total of 132,427 SNP markers was generated using the UNEAK. The resulting SNP data were filtered with a minor allele frequency greater than 0.05 and missing value less than 0.5. After filtering, the 47,275 SNP markers remaining were subjected to further analysis.

Linkage mapping and QTL analysis. Single-dose alleles (SDAs) of SNP markers with a ratio of less than 2:1 among F1 progenies were used to construct a genetic linkage map according to Li et al. (2014). Markers were grouped using JoinMap 4.1 with a minimum logarithm of odds (LOD) of 5. For each group, markers were clustered using default parameters. Linkage group (LG) numbers were assigned based on the alignment of alfalfa markers to the *Medicago truncatula* genome Mt V4.0 (<http://www.medicagohapmap.org/>). The results of linkage analysis were imported to the R package R/qtl v.1.44-9 (Broman and Sun 2009), and the function of plotmap was used to draw the linkage maps. Phenotypic data were analyzed using ANOVA. Least square means of three replicates were used for QTL mapping using the software of QTL IciMapping V4.1 (Meng et al. 2015). First, we integrated phenotypic data and genetic linkage map data to IciMapping. We then selected the build-in BIP (biparental populations) function and used the inclusive composite interval mapping with additive effect for QTL analysis. A permutation test was carried out using 1,000 parameters of permutation times. Type I error was 0.05. The LOD values were 3.0274 and 3.0349 for P1 and P2, respectively. Because the

permutation test standard was close to 3 in both parents, we used the value of LOD = 3 as the threshold to identify potential QTL. Other parameters were default. The resulting file of QTL analysis was obtained and imported to the software MapChart (Voorrips 2002) to display results.

Results

Phenotypic variations in plant responses to VW. Phenotyping VW resistance was evaluated using the foliar symptoms of individual plants inoculated with the pathogen in the greenhouse and scored for VW resistance. Resistance scores (1-to-5 scale) of three replications were analyzed for the 188 individuals (564 plants total; Supplementary Fig. S1). Phenotypic data were analyzed among the individuals using ANOVA (Table 1). The ANOVA showed significant differences ($P < 0.0001$) in phenotypic variation. The sums of squares were 800.37 and 94.15 for genotype and replicate, respectively. The *F* values of the ratio of the mean squares were 6.39 and 165.31 for genotype and replicate, respectively. The disease scores were normally distributed with P1 in the susceptible panel and P2 in the resistant panel (Fig. 1). The result for the Kruskal-Wallis test showed significant phenotypic variation among the F1 population ($P < 2.2e-16$), and resistance scores were tested for mean differences using the Dunn post hoc test. A heat map showing phenotypic significances was constructed using pairwise comparison between individuals of the F1 alfalfa population (Supplementary Fig. S1). Significant differences of grouping variable among the F1 individuals were observed. Representative groups containing 11 resistant individuals (A1, A11, A18, A2, A22, A27, A35, A44, A8, B126, and B137) and 18 susceptible individuals (A28, A4, A52, A61, A7, A72, A79, B12, B125, B34, A14, A21, A58, A69, B123, A56, B122, B11, and B15) are shown in Supplementary Table S1.

Construction of linkage maps. The genetic linkage map was constructed following the methods of Brouwer and Osborn (1999) and Li et al. (2014). Briefly, GBS SNP markers that were either polymorphic

Table 1. Analysis of variance of plant responses to *Verticillium* wilt in the F1 alfalfa population

Source	Degree of freedom	Sum of squares	<i>F</i> ratio	Prob > <i>F</i>
Genotype	187	800.37	6.39	<0.0001
Rep	1	94.15	165.31	<0.0001

Fig. 1. Phenotypic distribution of *Verticillium* wilt resistance of individuals in the full-sib population of alfalfa. Resistance scores obtained using a 1-to-5 scale, where 1 = susceptible (S) and 5 = resistant (R). P1 is susceptible parent 118S and P2 is resistance parent 58R. The top panel represents a box plot of mean and from three replicates.

only in P1 (55V50-118) with the parental configuration of AB $\times$ AA or polymorphic only in P2 (55V50-58) with parental configuration of AA $\times$ AB were screened for SDAs. Of those, the SDAs with a segregation ratio of 1:1 (presence/absence) were chosen. Because alfalfa is an autotetraploid species, a double-dose allele (e.g., AABB) is expected to have a 5:1 segregation ratio. Thus, alleles with segregation ratio less than 2:1 were considered to be SDAs. Using a cutoff value of 50% missing values, 5,010 SDA markers remained and were used for constructing linkage maps in both parents.

In all, 2,390 SDA markers were mapped into 32 LGs in the P1 parent and spanned a total length of 3042.54 centimorgans (cM) (Fig. 2A; Table 2). The average map density was 1.27 cM between markers. The number of markers in each LG ranged from 23 (LG 8D) to 144 (LG 3A). For the P2 parent, 2,620 markers were mapped into 32 LGs (Fig. 2B; Table 2) and they spanned 3,672 cM in length. The average map density was 1.40 cM between markers. The highest

number of markers (145) was mapped into LG 4C while the lowest marker density (22) was found in LGs 3B and 8D (Table 2).

QTL mapping. Two LOD scores were used in the QTL analysis. First, we used LOD 3 and identified five QTL associated with VW resistance in the two parents (Table 3; Figs. 3 and 4). Of those, one and four QTL were mapped in P1 and P2, respectively. The peak of *qVW-4B* was located near cumulative position 1,000 of the linkage map in LG 4B in P2 (Fig. 3A). The peak of *qVW-6B* was located near cumulative position 2,000 in LG 6B in P1 (Fig. 3B). The peaks of two QTL, *qVW-6D-1* and *qVW-6D-2*, were mapped in LG 6D in P2. They were located between cumulative positions 400 and 600 and positions 1,000 and 1,200, respectively (Fig. 3C). The QTL peak of *qVW-8C* was located between cumulative positions 3,000 and 4,000 in LG 8C in P2 (Fig. 3D). QTL *qVW-6B* was mapped on LG 6B between markers TP9940 to TP43550 (Fig. 4). Among four QTL identified in parent 2, *qVW-4B* was located between markers

Fig. 2. Genetic linkage maps of the F1 mapping population for two parents. Single-dose allele markers were used to construct genetic linkage map using JoinMap 4.1. The positions of markers are shown in Kosambi centimorgans (cM). Each linkage group has been assigned based on the *Medicago truncatula* genome with 8 chromosomes. Each chromosome has four haplotypes (A, B, C, and D).

TP75028 and TP94146, with a 3.13 LOD score (Table 3). Two QTL (*qVW-6D-1* and *qVW-6D-2*) were identified on LG 6D between markers TP55597 and TP61167, and TP59688 and TP136094, respectively. Their LOD scores were very close (4.85 and 4.86, respectively) (Table 3). The QTL *qVW-8C* was mapped on LG 8C between markers TP61627 and TP17963. This QTL had the highest value of LOD score (6.0) (Table 3).

To enhance the reliability of QTL, we increased the strength of LOD score to 4 and analyzed the same data set. Only three QTL remained when using LOD 4 as the threshold. They included two (*qVW-6D-1* and *qVW-6D-2*) on LG 6D and one (*qVW-8C*) on LG 8C. These QTL were more reliable because they were more significant and had higher LOD scores than the rest of the QTL identified using LOD 3 as threshold.

Table 2. Distribution of single-nucleotide polymorphism markers among 32 linkage groups of two parents for the F1 mapping population^a

Chromosome	Group	P1		P2	
		Markers (n)	Length (cM)	Markers (n)	Length (cM)
1	A	91	125.814	99	58.078
1	B	78	119.38	53	72.345
1	C	102	101.759	81	118.167
1	D	60	73.024	37	72.188
2	A	90	168.984	118	115.805
2	B	47	122.725	40	79.72
2	C	26	66.184	124	131.537
2	D	24	66.142	80	99.754
3	A	144	163.255	139	46.841
3	B	74	127.425	22	61.139
3	C	67	113.106	130	128.319
3	D	126	112.552	90	104.828
4	A	59	71.755	110	165.567
4	B	114	94.579	86	137.528
4	C	37	43.546	145	123.18
4	D	44	60.371	116	170.692
5	A	91	49.411	88	124.893
5	B	30	51.466	81	134.321
5	C	80	89.318	60	143.069
5	D	59	110.847	45	113.824
6	A	80	108.431	92	175.766
6	B	57	106.158	28	69.304
6	C	27	56.305	47	104.365
6	D	57	103.49	32	94.937
7	A	131	121.06	138	145.485
7	B	26	102.26	101	142.957
7	C	52	97.76	102	115.218
7	D	24	67.23	63	135.404
8	A	297	38.831	107	134.428
8	B	96	114.657	92	141.801
8	C	77	112.914	52	136.287
8	D	23	81.806	22	74.363
Total	...	2,390	3,042.545	2,620	3,672.11
Average	...	...	1.27	...	1.40

^a Length in centimorgans (cM).

Table 3. Quantitative trait loci (QTL) associated with Verticillium wilt resistance in the F1 population^a

Parent	QTL	LG	Pos	Left marker	Right marker	LOD	PVE (%)	Add	Candidate ^b
P1	<i>qVW-6B</i>	6B	2,063	TP90040	TP43550	4.43	3.87	0.78	...
P2	<i>qVW-4B</i>	4B	1,127	TP75028	TP94146	3.13	4.23	0.32	...
P2	<i>qVW-6D-1</i>	6D	495	TP55597	TP161167	4.79	5.6	0.36	...
P2	<i>qVW-6D-2</i>	6D	1,074	TP59688	TP136094	4	6.19	0.38	CC-NBS-LRR
P2	<i>qVW-8C</i>	8C	3,642	TP61627	TP17963	6	17.45	0.63	TIR-NBS-LRR

^a QTL were identified by inclusive composite interval mapping of additive effects. QTL, linkage group (LG), LG position (Pos), the left and right marker of the QTL, the logarithm of the odds (LOD), the percentage of the phenotypic variation (PVE) explained by QTL, and the additive effects (Add) of the QTL are presented.

^b Coiled-coil (CC) and toll interleukin 1 receptor (TIR) nucleotide binding site leucine-rich repeat (NBS-LRR).

Assignment of candidate genes associated with VW resistance loci. To identify possible candidate genes linked to the resistance loci, a genome browser was used against the *M. truncatula* genome sequences (Mt4.0 v1) at the Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>). Coiled-coil (CC) nucleotide binding site leucine-rich repeat (NBS-LRR) class and toll interleukin 1 receptor (TIR)-NBS-LRR class proteins were identified underlying the QTL intervals of *qVW-6D-2* and *qVW-8C*, respectively (Table 3). Both CC- and TIR-NBS-LRR genes are resistance genes and play roles in disease resistance in plants.

Discussion

In this study, we used a full-sib population consisted of 188 individuals developed from two parents with resistance or susceptibility to VW. Genotyping by sequencing resulted in 28,684 meaningful SNP markers. The UNEAK bioinformatics pipeline was used for genotype calling for the nonreferenced genome. SDAs of SNP markers generated more than 5,000 SDAs and they were used for constructing high-density linkage maps and mapping QTL for VW resistance. Five QTL were identified when the LOD score of 3 was used, whereas only three QTL remained using LOD 4. Two were located in LGs 6D and one in LG 8C. The two QTL (*qVW-6D-1* and *qVW-6D-2*) on LG 6D had LOD scores of 4.8. The QTL (*qVW-8C*) located in LG 8C had the highest LOD value (6.0). These QTL are more reliable and may contribute major effects to VW resistance. Two NBS-LRR genes were identified under QTL intervals of *qVW-6D-2* and *qVW-8C*. Given that the NBS-LRR gene family plays a major role in disease resistance in plants, they are likely candidates for VW resistance in the mapping population.

Genetic bases for VW resistance in alfalfa. Previous reports on the genetic basis of VW resistance in alfalfa suggested monogenic and quantitative resistance depending on the genetic background (Viands 1985). Our results showing multiple loci associated with VW resistance suggest quantitative resistance in the mapping populations. Miller and Christie (1991) reported that general combining ability significantly influenced the expression of resistance to VW in Vertus alfalfa and suggested that additive genetic variation is the primary source of VW resistance. This conclusion is supported by the results of the present study, in which multiple loci were identified for VW resistance.

Multiple QTL associated with VW resistance have been reported in the wild relative of alfalfa, *M. truncatula* (Negahi et al. 2014). They were located on LGs 1, 2, 6, and 8. Of those, two QTL located on LGs 6 and 8 were also found in *M. sativa* by the present study, although the additional QTL were located differently between the two species.

In this study of QTL associated with VW resistance, *qVW-8C* located on LG 8C showed the highest LOD score, suggesting its major contribution to VW resistance. This is consistent with our previous report, where most significant loci associated with VW resistance were identified on chromosome 8 in a panel of alfalfa breeding lines (Yu et al. 2016). Additional QTL identified on LGs 4 and 6 in the present study also was in agreement with the previous report (Yu et al. 2016). However, in the present study, two QTL (*qVW-6B* and *qVW-4B*) identified using LOD 3 were no longer significant when a higher strength of LOD 4 was used in the QTL analysis, suggesting that these QTL may be less reliable. It has been suggested that a LOD

Fig. 3. Profiles of quantitative trait loci (QTL) associated with Verticillium wilt resistance in the F1 population. The X-axis presents cumulative position in the linkage map, and Y-axis presents logarithm of the odds (LOD) scores for testing the likelihood of the linkage analysis. LOD score 3 was used as a threshold for QTL (solid line across genetic positions).

Fig. 4. Genetic map of quantitative trait loci (QTL) associated with alfalfa Verticillium wilt resistance detected using the full-sib population from a pair of resistant and susceptible parents: P1 = 118S and P2 = 58R. The R package R/qtl was used for QTL mapping and the bars represent mapping distance in centimorgans (cM). Rectangles indicate QTL regions with names of QTL on the right.

score of close to 3 should be used for QTL identification because it indicates that the chances of obtaining the observed data are 1,000 times more likely than if there are no QTL (Broman 2001). Our permutation test also showed that LOD 3 is the standard value in the QTL analysis. However, because the LOD score is a measurement of strength of evidence for the presence of a QTL at a genetic position, the use of higher LOD scores for QTL analysis would increase the evidence of QTL presentation. Therefore, the three QTL identified using higher LOD score in the present analysis would be more reliable.

The data that support the findings of this study are openly available in the NCBI Sequence Read Archive at <https://www.ncbi.nlm.nih.gov/sra/>, with the BioProject ID PRJNA343543.

Putative candidate genes underlying QTL intervals for VW resistance. The two NBS-LRR genes underlying the QTL intervals of *qVW-6D-2* and *qVW-8C* are resistance genes and could be candidates for conferring resistance to VW in the mapping population. This is consistent with our previous study where two TIR-NBS-LRR genes associated with VW resistance were identified in a breeding population of alfalfa by GWAS (Yu et al. 2016). They were also located on the same chromosome, 8. The plant NBS-LRR gene family contains a large class of disease resistance genes. The TIR-NBS-LRR genes play roles in disease resistance in plants. There is evidence that the TIR-NBS-LRR gene (*RCT1*) from *M. truncatula* confers resistance to anthracnose in transgenic alfalfa plants (Yang et al. 2008). The identification of the TIR-NBS-LRR genes in previous reports and in the present study suggests their contribution to VW resistance in alfalfa. The identification of multiple NBS-LRR genes confirms that VW resistance might be controlled by polygenic inheritance in alfalfa.

Although a good agreement of the resistance loci was found between different populations in different studies, further validation of markers identified is needed before they can be used for MAS. We plan to test all significant markers identified in this and previous studies in a wide range of populations with known background of resistance to VW in alfalfa. Efforts aimed at development of a high-throughput SNP genotyping platform for a comprehensive genome scan of segregating populations combined with additional phenotypic data for VW response will be used to validate the markers. VW resistance loci identified in this study increase the likelihood that quantitative resistance contributes to VW resistance in alfalfa. In the future, obtaining additional information about the candidate genes underlying the QTL intervals may provide insight into the VW resistance gene network in *Medicago* spp. and accelerate breeding process for new alfalfa cultivars with improved resistance to VW.

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