Mapping and Validation of Resistance to *Aphanomyces euteiches* in Alfalfa

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INTRODUCTION

Aphanomyces euteiches is an economically important soilborne oomycete pathogen of alfalfa (*Medicago sativa*) in North America. The pathogen persists in soil organic matter and infects seedlings in prolonged moist conditions, causing root rot, reduced growth, chlorosis, and death. Two races (Race 1 and 2) have been identified and are widespread across Midwestern dairy states and Canada. Genetic resistance is available for both races of *A. euteiches* and heritability for resistance to both is high, ranging from 0.6 to 0.9. As a result, adequate resistance has been developed in many commercially available alfalfa varieties. These varieties have exhibited improved seedling health, forage yield, and stand persistence under field conditions favoring *A. euteiches*. However, the high resistance rating (HR) from an industry standard test only requires greater than 50% of plants with resistance. Higher thresholds for resistance have been a challenge to achieve, possibly due to synthetic breeding methods typically utilized in autotetraploid alfalfa. If certain resistance genes exhibit dominance, they mask three susceptible alleles under selection pressure, which are then exposed in the absence of selection pressure as seed of synthetic varieties is produced for market.

Advances in genetic mapping tools for polyploids have been rare but highly valuable. SNP arrays have been created for several polyploid crops and software for making automated genotype calls on polyploid SNP data also exists. For alfalfa, Li et al. (2014) have developed an array of ~8,000 SNP's that are publicly available. Genetic analysis of autotetraploid potato by Rosyara et al. (2016) and associated software developed for potato are directly applicable to autotetraploid alfalfa. Furthermore, the complete alfalfa genome has been sequenced by Chen et al. (2020), making it possible to explore any chromosome region of interest for candidate genes based on trait-associated molecular markers. The objectives of this study were to map QTL for resistance to *A. euteiches* in elite alfalfa germplasm, validate QTL through confirmed high levels of resistance, and enable MAS in cultivar development which could achieve higher levels of resistance in the case of dominance than phenotypic selection alone.

METHODS

Phenotypic Data Collection

A modified seedling straw test was used to generate phenotypic data. An isolate of *A. euteiches* race 2 (APH2) designated as WAS-5 was grown on petri plates of potato dextrose agar (PDA) for one week at 26°C. A 10µl Eppendorf pipette tip was used to cut a plug of PDA containing the fungal mycelium and the pipette tip was then placed over a cut seedling with the stem immersed in the PDA plug (Fig 1a). Inoculated flats of seedlings were placed under plastic domes in dim lighting at 22°C for 72 hours, then domes and pipette tips were removed. After 7 days the seedlings were returned to 14 hours full lighting and after 14 days each seedling was inspected for the presence of a lesion on the cut internode. Necrosis of most or all of the internode was scored as susceptible and internodes remaining green were scored as resistant (Fig 1b).

Genotyping and QTL Mapping

Prior to cutting and inoculation, four leaflets from each seedling of four biparental populations were transferred, using forceps, to a 96-well plate for DNA extraction at the Corteva High Throughput Genotyping Laboratory in Johnston, IA. The number of seedlings able to be tissue sampled for each population were A142156/C16-432 (121), A241/C17-513 (122), A241/C15-0414 (122), and A20-4033/C15-0817 (138). Genotyping was conducted using single nucleotide polymorphism (SNP) markers in a custom designed Infinium array (Illumina) of ~8000 publicly available SNP's, created by Corteva Agriscience. Reported SNP positions in *M. truncatula* were utilized for mapping and QTL detection was performed using both additive and dominance models with the R package GWASpoly.

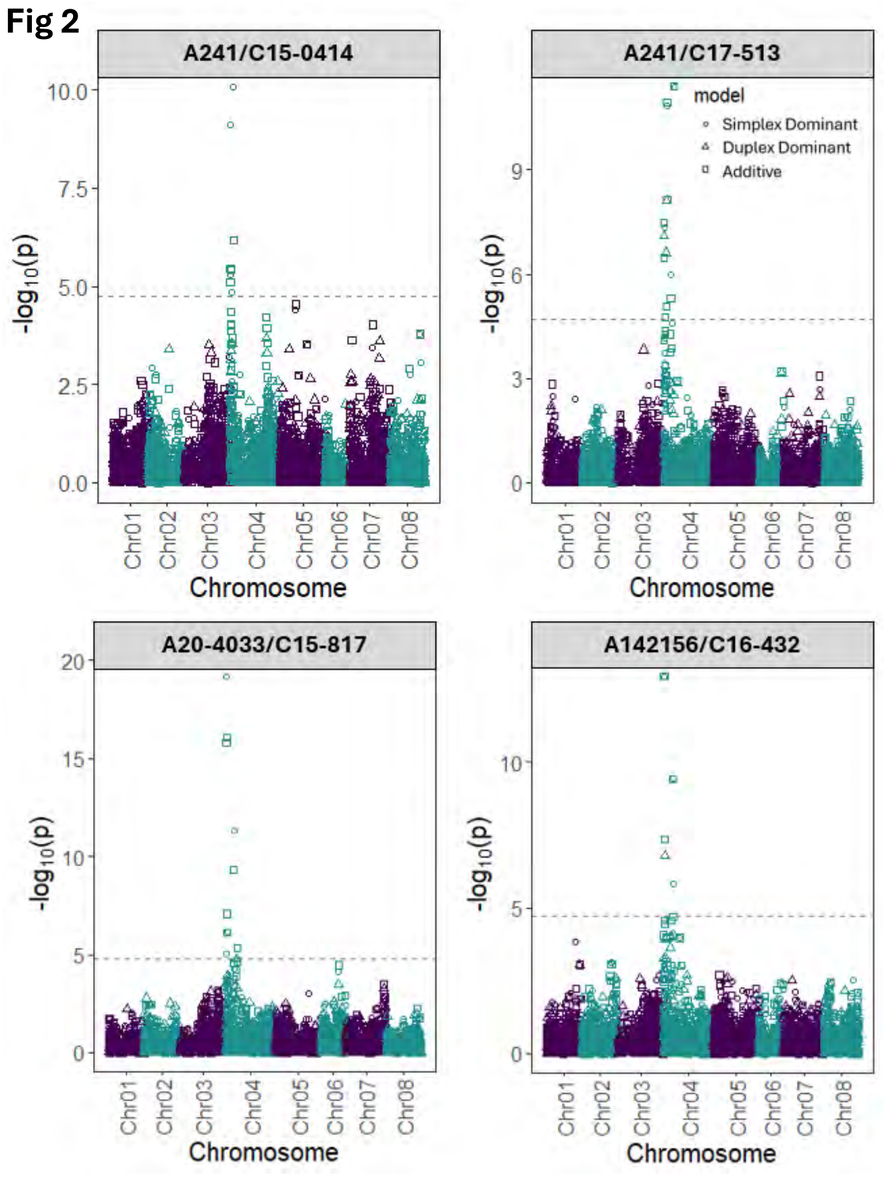
Fig 1a

Fig 1b

RESULTS

APH2 resistance was detected for 53 of 121 seedlings (44%) in the A142156/C16-432 population, 53 of 122 seedlings (43%) in the A241/C17-513 population, 97 of 122 seedlings (80%) in the A241/C15-0414 population, and 70 of 138 seedlings (51%) in the A20-4033/C15-0817 population. Additive, simplex dominant, and duplex dominant models from GWASpoly analysis all detected markers on chromosome 4 above LOD score thresholds which ranged from 4.70 to 4.75 (Fig 2). Most simplex dominant model LOD scores were equal to or higher than corresponding additive model scores. Twenty significant marker effects across all four populations were above the respective LOD thresholds and map to a 20 million base-pair region on chromosome 4.

Fig 2



In previous unpublished observations, APH2 resistance in alfalfa has exhibited dominant inheritance and high heritability. The expected segregation ratio (1:1) for a dominant gene in an autotetraploid biparental cross was also reflected in three of the four mapping populations (discussion on fourth mapping population segregation ratio below). These observations, along with classical gene-for-gene theory of interaction between plant hosts and pathogen races, lead to the hypothesis that a major, simply inherited resistance gene (R-gene) exists in alfalfa for APH2. In addition, Ameline-Torregrosa et al. (2007) report a cluster of nucleotide-binding site Leucine-rich repeat (NBS-LRR) genes on chromosome 4 of *M. truncatula*. A search of the annotated medsa.XinJiangDaYe *M. sativa* genome assembly reveals this NBS-LRR gene cluster in the syntenic 20 million base pair region identified by our significant markers. These findings are also supported by preliminary results from mapping APH2 resistance in the public variety and industry standard check, WAPH-5, by Samac et al. (2024). Table 1 combines the key reported DaRT marker position from the WAPH-5 study, the starting bp position of our markers, and the starting bp position of numerous NBS-LRR genes on chromosome 4 of *M. sativa*.

Table 1

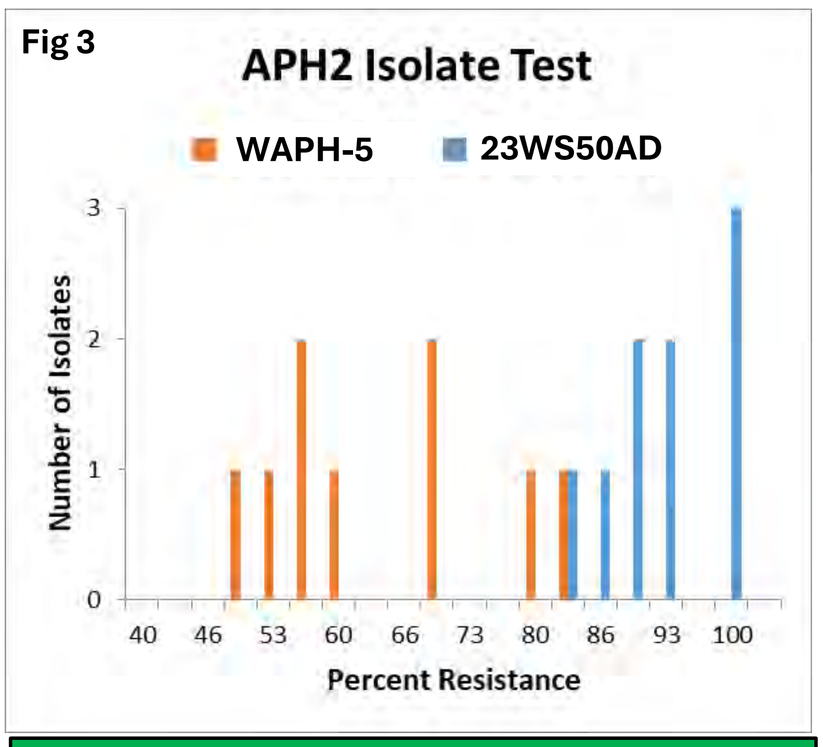
SNP or gene	M. sativa Chr4.1	
S_15342922	70,644,552	
S_9141167	71,132,781	← qPCR Assay Developed
S_8557330	71,574,430	
S_7552767	74,136,693	
S_8884216	75,759,978	
DaRT_1374366	80,478,320	← WAPH-5 QTL Peak (Samac, 2024)
NBS-LRR gene	81,267,912	
NBS-LRR gene	81,562,968	
NBS-LRR gene	81,751,894	
NBS-LRR gene	81,885,910	
NBS-LRR gene	81,926,798	
NBS-LRR gene	82,044,176	
NBS-LRR gene	82,110,915	
NBS-LRR gene	82,124,222	
NBS-LRR gene	82,136,485	
NBS-LRR gene	82,179,313	
NBS-LRR gene	82,190,181	
NBS-LRR gene	82,220,956	
NBS-LRR gene	82,295,457	← NBS-LRR Cluster (Ameline-Torregrosa et al, 2008)
S_10108080	84,163,501	
S_24248838	84,173,012	← qPCR Assay Developed
S_24560229	85,652,501	
NBS-LRR gene	85,863,756	
S_18557967	87,535,923	
S_24908062	88,443,672	
S_587216	88,698,134	
S_19246557	89,062,535	
S_23642916	89,112,808	
S_23641495	89,113,306	← qPCR Assay Developed
S_22092534	89,176,482	
S_23545123	89,287,385	
S_20025557	89,494,749	

VALIDATION

The segregation ratios for three of the mapping populations were close to 50% resistant and 50% susceptible, the expected ratio (1:1) for a simplex dominant by wildtype cross. However, the A241/C15-0414 population was 80% resistant. This segregation ratio is close to the expected duplex by wildtype ratio of 83% resistance (1D:4S:1N). The population size of A241/C15-0414 of 122 seedlings would yield an expected 20 duplex plants. Inspection of the linked marker genotypes in the A241/C15-0414 population resulted in selection of eight surviving, putative duplex progeny plants from this cross. These plants enabled both the validation of the R-gene discovery and a proof of concept for higher levels of resistance in alfalfa products. These plants were polycrossed to create a SYN1 seed lot (23WS50AD) where the expected segregation of resistance alleles would be 1Q:8T:18D:8S:1N.

The SYN1 seed of 23WS50AD was sown in a replicated APH2 isolate test along with industry standard checks WAPH-1, WAPH-5, and Saranac in a growth room at West Salem, WI. Growing conditions were 14 hours of light and 22°C. The test used nine isolates of APH2 designated as WAS-4, WAS-5, OLD-3, OLD-6, S6-DS, RAD-3, NC-1 DR, S9, and ELK-8. According to the industry standard test protocol, seedlings were grown in vermiculite, inoculated with comminuted mycelium, maintained in flooded conditions, and then scored for resistance. The results show consistent performance of 23WS50AD at resistance levels between 80% and 100% for all isolates (Fig 3). Resistance levels of the industry standard check for resistance (WAPH-5) ranged from ~50% to ~80%. Resistance levels of susceptible checks (WAPH-1 and Saranac) were at or near 0% (not shown).

Fig 3



CONCLUSIONS

The findings of this study show that a highly effective R-gene on chromosome 4 of *M. sativa* confers resistance to APH2, a serious seedling disease in hay production areas. This resistance allele has been selected through conventional breeding for many breeding cycles, however, due to dominance, synthetic generations of seed increase unmask three recessive susceptible alleles. By using SNP data to identify duplex dominant plants, we show the ability of MAS to overcome the problem created by dominant inheritance of this R-gene. A synthetic created with duplex dominant plants carries only two recessive susceptible alleles, greatly improving the overall resistance level in subsequent synthetic generations. In our isolate test, WAPH-5 performed as a typical highly resistant (HR) rated variety, with a normal distribution of isolates averaging ~65%. Given that Samac et al. show the same R-gene exists in WAPH-5, this result represents the typical resistance levels achieved with conventional breeding which selects for this R-gene. The advantage of the MAS derived 23WS50AD against the same isolates is evident in skewing toward 100% resistance. These higher levels are due to the much higher allele frequency in this synthetic variety than is possible with phenotypic mass selection. Lastly, it is worth noting that others have interpreted variation in the virulence of numerous isolates of APH2 as evidence of a new race of this pathogen. Given that the R-gene on chromosome 4 is highly effective against all APH2 isolates tested, we find no evidence of a new race of *A. euteiches*.

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SPECIAL THANKS

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