Brief Communication

Two linked resistance genes function divergently in defence against Verticillium Wilt in Alfalfa

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Introduction

Verticillium wilt (VW) is a soil-borne fungus disease in a wide range of plant species including alfalfa and caused yield loss up to 50% in less than 3 years due to the rapid spreading of the pathogen in soil (Vandemark et al., 2006). However, the genes responsible for resistance against V. alfalfa is still unknown. In tomato, a well-studied gene locus for VW resistance is known as Ve (Fradin et al., 2011). However, the orthologues in M. truncatula and M. sativa share low sequence identity with the tomato Ve1 and were not involved in the response to VW (Toueni et al., 2016), indicating a different mechanism of VW resistance in Medicago.

In our previous study, we reported several markers associated with VW resistance in alfalfa. One of them was located on chromosome 8 and showed major effect on VW resistance (Yu et al., 2017). In the present study, we identified two candidate genes, MsVR38 and MsVR39 localized at the same locus and linked each other in a head-to-tail orientation. The gap between these two genes was < 3 kb (Figure 1a). The genomic sequence of MsVR38 was 8.4 kb, which was 1.2 kb longer than MsVR39. Both MsVR38 and MsVR39 contained five exons (Figure 1b). Annotation of MsVR38 and MsVR39 found that both were members of the Toll/Interleukin1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NBS-LRR) gene family, and they both contained 14 LRR repeats (Figure 1c). The alignment of protein sequences showed nearly 90% identity between MsVR38 and MsVR39 (Figure 1d), while < 27% identity was found when comparing them with tomato Ve1 (Figure 1e). We are interested in learning if both MsVR38 and MsVR39 play the same role in response to the VW disease in alfalfa.

To assess the functions of MsVR38 and MsVR39 on VW resistance, we selected resistant and susceptible individuals, R384 and S371, respectively, from 317 alfalfa breeding lines and performed bioassay using V. alfalfa. The responses of plants to V. alfalfa showed that R384 had greater resistance compared with S371 (Figure 1f). We cloned and compared CDS region of MsVR38 and MsVR39 between S371 and R384. A missing nucleotide was identified in MsVR38 of R384, which means MsVR38 in R384 was disrupted and not expressed normally (Figure 1g). Then, we inoculated alfalfa leaves of S371 and R384 with Czapek-Dox medium containing exproteins from V. alfalfa to analyse gene expression in response to the infection of V. alfalfa. The leaves from S371 displayed more severely wilt syndrome compared with those from R384 after inoculation (Figure 1h). Transcriptomic results suggested that, comparing to MsVR38, the expression of MsVR39 was significantly increased in both S371 and R384, implying the positive role of MsVR39 against V. alfalfa (Figure 1i). This result was further confirmed by qPCR (Figure 1j). The relative expression level of MsVR39 in the control of R384 was even higher than that in S371 after inoculation, which could be one of reasons that render R384 greater resistant than S371.

Next, we identified MtVR130 and MtVR140, the homologs of MsVR38 and MsVR39, respectively, from the M. truncatula genome, a model plant of close relative to M. sativa to further verify their function on VW resistance. Comparable to M. sativa, MtVR130 and MtVR140 were also localized on chromosome 8 with the distance less than 3 kb between each other. More importantly, the pair of R genes are highly identical in M. sativa and M. truncatula (Figure 1k). MtVR130 shared 96% similarity with MsVR38, and MtVR140 shared 93% similarity with MsVR39, implying the conservative function of the R gene pair between the two species.

Wildtype M. truncatula R108 and two Tnt1 insertion mutants: MtVR130 and MtVR140 were used for further function assessment. The phenotype of infected plants demonstrated that MtVR130 was more resistant, whereas MtVR140 was more susceptible to VW compared with the wildtype R108, suggesting that disruption of MtVR130 increased the resistance to V. alfalfa in M. truncatula. (Figure 1l). The results of fungal recovery assay also demonstrated a higher resistance of MtVR130 (Figure 1m). Collectively, our results revealed that MtVR130 plays negative role in regulation of the disease response, whereas MtVR140 is a positive R gene on defending against *Verticillium*.

A question raised by above results is why the pair of R genes shares high identity but functioned differently on defending against *Verticillium*. Earlier studies suggested that self-association of TIR domain is essential for the function of resistance signalling (Zhang et al., 2017). It would be interesting to know whether the TIR association exists between MsVR38 and MsVR39 because of their high-identity sequences (Figure 1n). To answer the question, we cloned the TIR coding sequences from MsVR38 and MsVR39 and ligated them into pDEST32 (baits) and pDEST22 (preys) plasmids for interaction analysis using yeast two hybrid. The results demonstrated that either the TIR domains in MsVR38 or MsVR39 could self-associate to form homodimers. However, the interaction between TIR from MsVR38 and MsVR39 was stronger than those of homodimers, indicating that when MsVR38 and
MsVR39 both normally expressed, MsVR38 and MsVR39 were more inclined to form into heterodimers, which could negatively regulate the response of Verticillium wilt resistance (Figure 1o).

The discrepancy of binding affinity might be resulted from the major unaligned region in TIR domains of MsVR38 and MsVR39 as shown in Figure 1n.
Plant R genes were considered to confer resistance against pathogens, only few of them govern the susceptibility to plant disease (Lorang et al., 2007). In this study, we found that one of the TIR-NBS-LRR genes, MsVR39 responded positively to VW in *M. sativa* while negative effect was observed on the other gene MsVR38. Further investigation on the *M. truncatula* mutants showed that knockout of MtVR130, the homologue of MsVR38 provided a greater resistance against VW compared with the wildtype. Conversely, mutant of MtVR140, the homologue of MsVR39 was more susceptible. A possible mechanism is that when the pair of highly identical TIR-NBS-LRR genes are both normally expressed, they are more likely to form heterodimers, which inhibits the formation of MsVR39 homodimers, thus causing susceptibility to VW disease.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

LXY and SL designed the experiment. SL and LXY wrote and edited the manuscript. SL and YN performed experiments. SL and CAM analysed the data.