Developing Powdery Mildew Resistance in Hop Through CRISPR-Mediated Mutagenesis of *MLO S*-genes

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Abstract

Hop (*Humulus lupulus*) faces substantial yield and quality losses due to powdery mildew (PM), caused by *Podosphaera macularis*. Targeting susceptibility (*S*) genes, such as *Mildew Locus O* (*MLO*), via CRISPR-mutagenesis, offers a promising strategy for durable PM resistance. We aimed to develop a transformation and CRISPR/Cas9 editing system in hop to enable targeted mutagenesis of *MLO S*-genes. First, we characterized *MLO* homologs from the 'Cascade' assembly, focusing on Clade V *MLO*s which are commonly associated with disease susceptibility. Guide RNAs targeting two hop *AtMLO12* homologs were delivered to *in vitro*-derived tissue of 'Fuggle' via *Agrobacterium*-mediated transformation. After 6 to 12 weeks on regeneration media, a small percentage of marker positive edited shoots were recovered. Many edits were small indels causing premature stop codons and truncation of the C-terminal calmodulin-binding domain. To date, at least six independent CRISPR-edited transformation events have been recovered. Preliminary phenotyping of *in vitro*–derived leaves revealed variable resistance and pleiotropic effects, including chlorosis, early senescence, and reduced vigor. These findings support our hypothesis that these *AtMLO12* homologs play a role in powdery mildew susceptibility and demonstrate the feasibility of genome editing in hop. Ongoing work aims to minimize pleiotropy while maintaining *mlo*-mediated powdery mildew resistance.

Key words. CRISPR, MLO, powdery mildew

Introduction

Powdery mildew causes significant economic losses by reducing both yield and cone quality. To mitigate these impacts and avoid issues related to export compliance with fungicide maximum residue levels, we are exploring the use of nullified susceptibility genes (*S*-genes) as a strategy to confer broad-spectrum resistance to powdery mildew. *S*-genes were first discovered in barley in 1942, when powdery mildew resistance was observed following X-ray mutagenesis. This resistance was subsequently linked to loss-of-function mutations in the *Mildew Locus O* (*MLO*) gene, an *S*-gene necessary for host susceptibility (JØRGENSEN 1992). Functional *MLO* alleles confer host susceptibility to compatible pathogen interactions, while loss-of-function (nullified) *mlo* alleles result in incompatible interactions and resistance. The haplotype-phased 'Cascade' reference genome contains at least 20 RNA-seq supported *MLO* homologs, four of which fall within Clade V—the clade most frequently associated with broad-spectrum resistance upon gene nullification (Table 1)(KUSCH et al. 2016). Functional characterization of these putative *S*-genes is needed to definitively establish their role in powdery mildew susceptibility in hop.

Prior to functional characterization, establishing a robust transformation, regeneration, and genome-editing protocol is necessary, as hop is notoriously recalcitrant. Previous studies report that regeneration rates via *de novo* shoot organogenesis vary widely between genotypes (e.g. BATISTA et al. 1996; LIBERATORE et al. 2020). There are sparse reports of successful transformation, regeneration, and editing in hop with those demonstrating efficiency rates between 0% and 3% (AWASTHI et al. 2021; SIMS 2022). In this work, we aim to establish a protocol for transformation, editing, and regeneration in hop. We design, mobilize, and deliver

CRISPR/Cas constructs with guide RNAs targeting putative *MLO S*-genes via *Agrobacterium*-mediated transformation. We also report ongoing evaluation of the resulting transgenic lines through phenotyping for powdery mildew resistance and plant vigor, alongside allele-specific genotyping of the targeted loci.

Gene ID	Scaffold	Start	AA Length	UniProt Homolog
HUMLU_CAS0068957	Scaffold_49	230084079	552	MLO12_ARATH
HUMLU_CAS0050357	Scaffold_77	171879632	530*	MLO6_ARATH
HUMLU_CAS0048594	Scaffold_77	62805110	533	MLO12_ARATH
HUMLU_CAS0050448	Scaffold_77	17509059	605	MLO6_ARATH

Table 1. Putative *Arabidopsis* MLO *S*-gene homologs in hop.
*One haplotype of HUMLU_CAS0050357 appears to have a 5' truncated variant in Cascade (345 AA).

Material and methods

MLO homologs in hop were identified by BLASTing amino acid sequences of *Arabidopsis thaliana MLO2*, *MLO6*, and *MLO12* against the 'Cascade' Dovetail genome assembly (PADGITT-COBB et al. 2023). Candidate sequences were further validated based on predicted protein domain architecture, presence of conserved motifs, and RNA-Seq expression profiles. Guide RNAs were selected using Geneious Prime's "Find CRISPR Sites" tool, prioritizing candidates with high predicted activity and low probability of off-target effects (DOENCH et al. 2016). These candidate gRNAs were delivered within a CRISPR-Cas9 system as outlined in **Figure 1**.

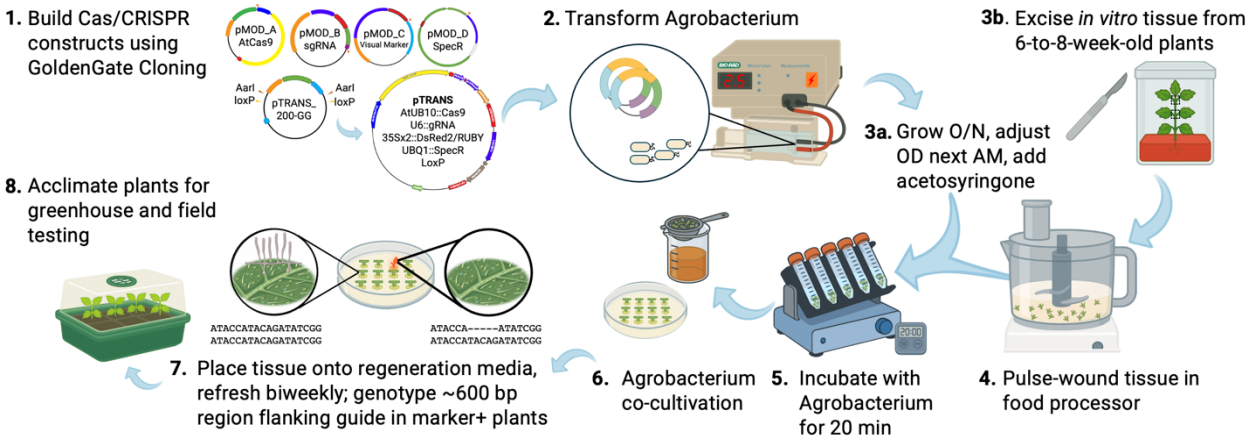

Figure 1. General approach for transformation and CRISPR-Cas9 editing in hop. **(1)** CRISPR constructs were assembled via Golden Gate cloning using customized components based on the Voytas Lab plant bioengineering toolkit (ČERMÁK et al. 2017). Constructs were verified by Nanopore sequencing, **(2)** transformed into *Agrobacterium* strain AGL-1, and **(3a)** cultured overnight at 28°C with shaking at 125 rpm. Following centrifugation, AGL-1 cultures were resuspended in induction media and adjusted to OD_{600}=0.6. **(3b)** Tissue from six-to-eight-week-old *in vitro*-grown 'Fuggle' hop plants was excised and then **(4)** pulse-wounded in induction medium using a food processor. Tissues were then **(5)** incubated with *Agrobacterium* on a rocker for 20 minutes. Following incubation, tissues **(6)** were transferred to a co-cultivation medium for 3 days. After co-cultivation, explants were washed, briefly dried, and placed on regeneration medium. **(7)** Explants were transferred biweekly onto fresh media and maintained

at 24°C under broad-spectrum fluorescent lighting (40 µmol m^{-2} s^{-1}) with a 16-hour photoperiod. DNA from DsRed-positive shoots was amplified (~600 nucleotides surrounding PAM site) and sequenced via Sanger and Nanopore sequencing. Editing efficiency was assessed on haplotypes ± 20 nucleotides from the guide PAM site using the CRISPR editing efficiency tool in Geneious and with DECODR (https://decodr.org/). Susceptibility to *P. macularis* was assessed by inoculating leaf disks (1 cm diameter) from the youngest fully unfurled leaves of *in vitro*-grown edited and wild-type plants using a settling tower. Percent disease severity was assessed 10 days later using a powdery mildew trained computer vision model. **(8)** Edited plants were acclimated to greenhouse conditions over a 3-week period following root development *in vitro.*

Results

To date, at least six independent transformation and editing events have been recovered, with five lines successfully surviving the acclimation process. All edited plants were produced with a guide designed to target the two AtMLO12 clade V homologs (Table 1) and thus four loci. The edited lines displayed a range of genotypes, including biallelic heterozygous, biallelic homozygous, monoallelic, and chimeric variants, predominantly featuring small insertions or deletions (<5 nucleotides) occurring a few bases from the PAM site. Many of the observed edits introduced a premature stop codon between amino acid positions 310 and 370 (out of 533 or 552 total), resulting in a truncated protein that lacks the C-terminal calmodulin-binding domain—an essential region for MLO function. Some lines presented pleiotropic effects *in vitro* including early senescence, chlorosis, stunting, and reduced vigor (Figure 2C). Preliminary inoculations of *in vitro*–derived leaf disks with *P. macularis* isolates revealed a spectrum of phenotypic responses, ranging from complete resistance to marginal reductions in disease severity (example Figure 2A, 2B).

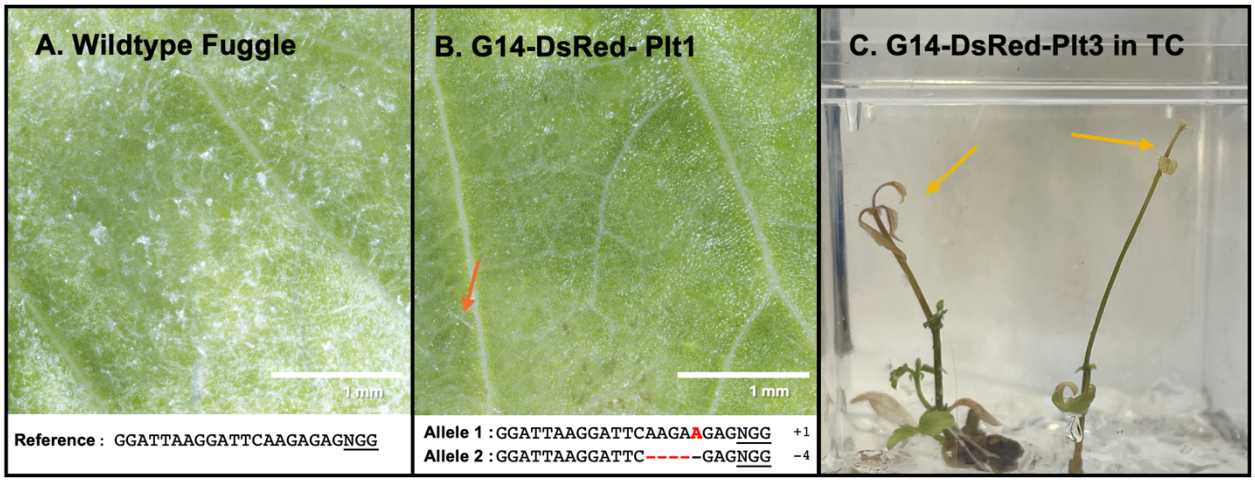

Figure 2. (A, B) Representative comparison of powdery mildew susceptibility between wild-type 'Fuggle' and a biallelic heterozygous KO-*MLO* line 10 days post inoculation. Limited sporulation is present on the edited line (red arrow), a recurring observation with inoculated *in vitro* leaf disks from edited lines. **(C)** Example of early senescence as a potential pleiotropic effect in tissue culture (TC), more frequently observed in certain edited lines (yellow arrows).

Discussion

This study demonstrates the successful targeted mutagenesis of two *AtMLO12*

homologs in hop, resulting in a spectrum of powdery mildew resistance phenotypes across edited lines tested *in vitro*. The observed increase in resistance in some lines supports the role of these *MLO* homologs as susceptibility genes in hop, consistent with findings in other species. Since all phenotyping data currently originate from *in vitro*–derived tissue, we anticipate differences in susceptibility once plants are in greenhouse or field conditions.

The observation of pleiotropic effects also highlights the trade-offs involved in *mlo*-based resistance. As C-terminal calmodulin binding on MLOs is thought to play an autoinhibitory role in calcium transport, it is possible the pleiotropic effects we are observing are due to unregulated Ca^{2+} movement. Unregulated Ca^{2+} influx can disrupt cellular homeostasis by overactivating calcium-dependent signaling pathways, including those involved in defense, oxidative stress, and programmed cell death. In the absence of calmodulin-mediated gating, excessive calcium may lead to constitutive activation of immune responses, resulting in ROS accumulation.

We are exploring alternative targets and editing strategies that retain the beneficial powdery mildew resistance conferred by *MLO* knockouts while reducing associated pleiotropic effects. Ultimately, our goal is to achieve this using a transgene-free or excisable genome editing system; however, confirming the functional roles of candidate *MLO* genes remains a critical first step in the process.

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