Hygromycin resistance gene screening

PCR primer set and reaction conditions used for screening regenerated T_0 plantlets for the presence of hygromycin resistance gene.

Primers:

HygFw 5' ACTCACCGCGACGTCTGTCG 3'
HygRv 5' GCGCGTCTGCTGCTCCATA 3'

PCR conditions:

96 °C for 2 min
40 cycles of
    96 °C 45 sec
    60 °C for 30 sec
    72 °C for 45 sec
72 °C for 2 min
4 °C storage

Mlo mutation screening

PCR primer set, PCR reaction conditions, as well as sequencing primers used for *Mlo* mutation screening.

Primers:

MloFw 5' TGGTGCTCGTGTCCGTCCTCAT 3'
MloRv 5'GCCACCTTGCCCTGCAAACACA 3'

PCR conditions:

98 °C for 2 min
35 cycles of
    98 °C for 20 sec
    60 °C for 30 sec
    72 °C for 30 sec
72 °C for 3 min
4 °C storage

Sequencing primer for *Mlo* mutation screening amplicon 5' TGGTGCTCGTGTCCGTCCTCAT 3'
Sequencing primer for TOPO cloned amplicon (T3 primer) 5' ATTAACCCTCACTAAAG 3'

PCR for creating bacterial and fungal libraries

PCR reaction conditions for amplifying the V3/V4 region of the 16S rRNA and the ITS2 region of the fungal rRNA.

PCR amplifications were performed in a 25 μL reaction mixture, with GoTaq Flexi polymerase (Promega Corporation), according to the supplier's manual.

PCR conditions:

25 cycles of
    94 °C for 30 sec
    55 °C/57 °C (bacteria/fungi) for 30 sec
    72 °C for 30 sec
72 °C for 10 min

CasOFFinder off-target analysis

CasOFFinder analysis was performed for the designed spacer sequence to ensure there are no obvious off-target risks associated with our genome editing approach. The analysis allowed for one mismatch and one bulge. Results are presented in Figure S1.

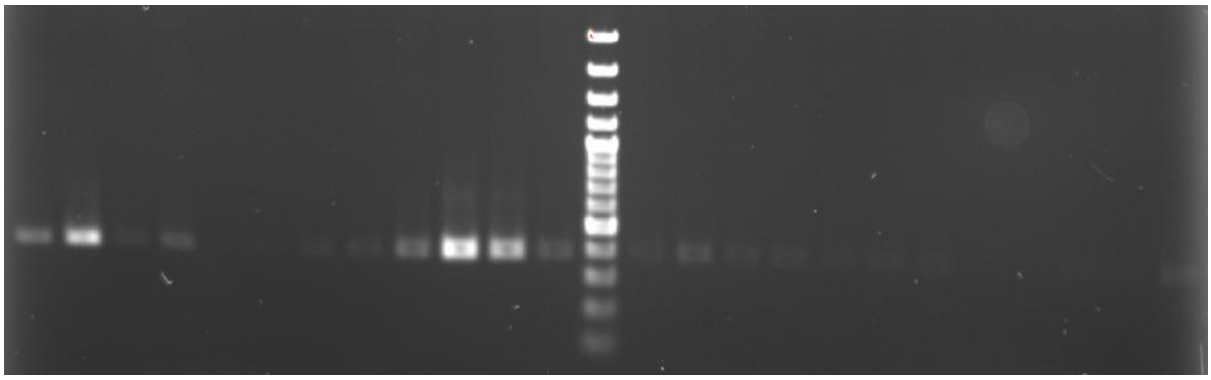
```
#Bulge type     crRNA    DNA     Chromosome      Position        Direction       Mismatches      Bulge Size
X       CAAGTACGTTGACTACTGCCNGG CAAGTACGTTGACTACTGCCCGG chr4    521980797       -       0       0
DNA     C-AAGTACGTTGACTACTGCCNGG        gCAAGTACGTTGACTACTGCCCGG        chr4    521980797       -       1       1
RNA     CAAGTACGTTGACTACTGCCNGG a-AGTACGTTGACTACTGCCCGG chr4    521980797       -       1       1
RNA     CAAGTACGTTGACTACTGCCNGG aA-GTACGTTGACTACTGCCCGG chr4    521980797       -       1       1
```

Figure S1. CasOFFinder off-target analysis results for the designed spacer. All hits correspond to the *Mlo* gene itself. Hence, there was no potential off-target.

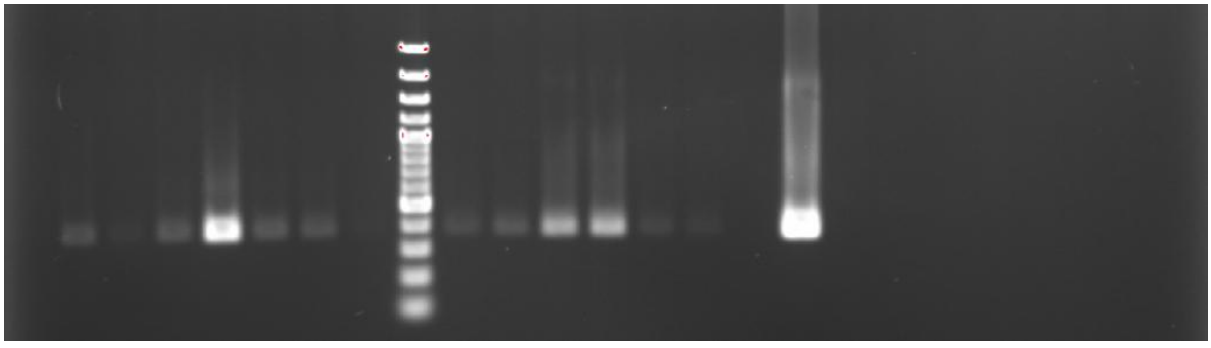
Hygromycin resistance gene and targeted region screening

(A)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
#1	#5	#14	#15	#16	#17	#21	#22	#3	#4	#6	#7	L	#9	#10	#11	#12	#20	#34	#35	#37	#38	#40	#41	#42



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
#25	#26	#27	#28	#31	#32	#43	#44	L	#13	#18	#23	#24	#29	#30	-	+	WT	X	X	X	X	X	X	X



(B)

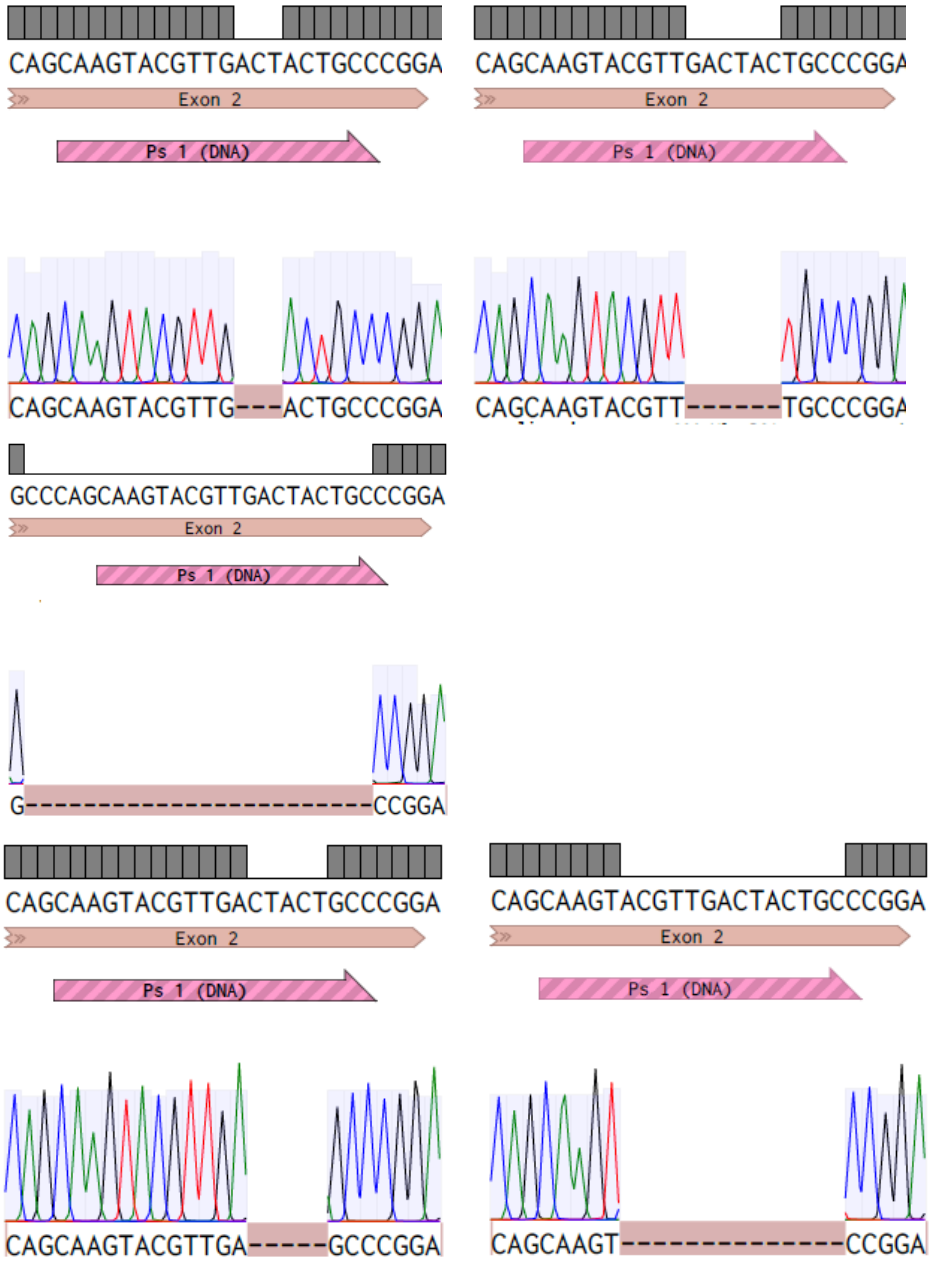


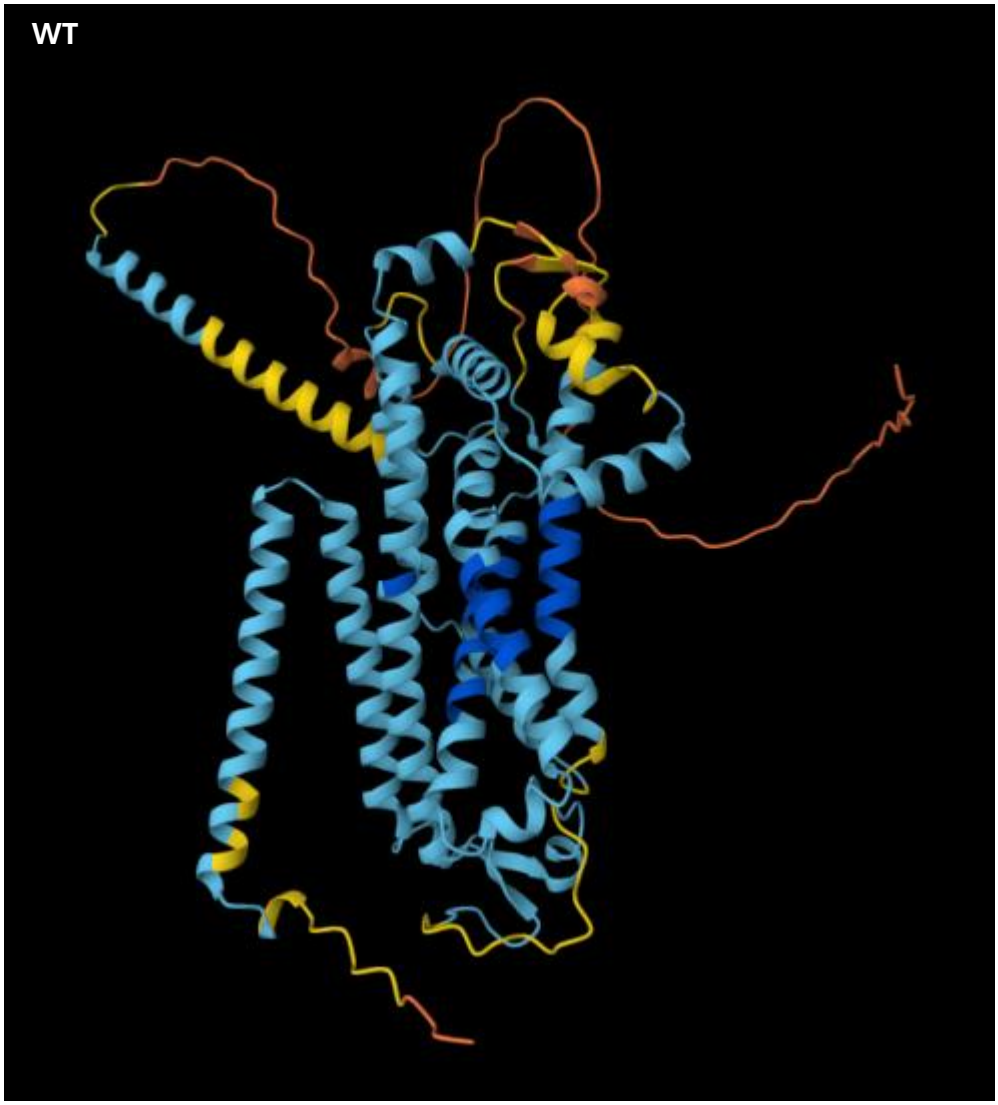
Figure S2. Hygromycin resistance gene screening results in T0 plants. The table above the gel indicates the identity of the T0 plant loaded in each well. L, 100+ DNA ladder; − and +, negative and positive controls; WT, wild-type cultivar 'Maythorpe'; X, no sample. (B) Sequencing chromatograms of the genotypes used in the resistance scoring experiments.

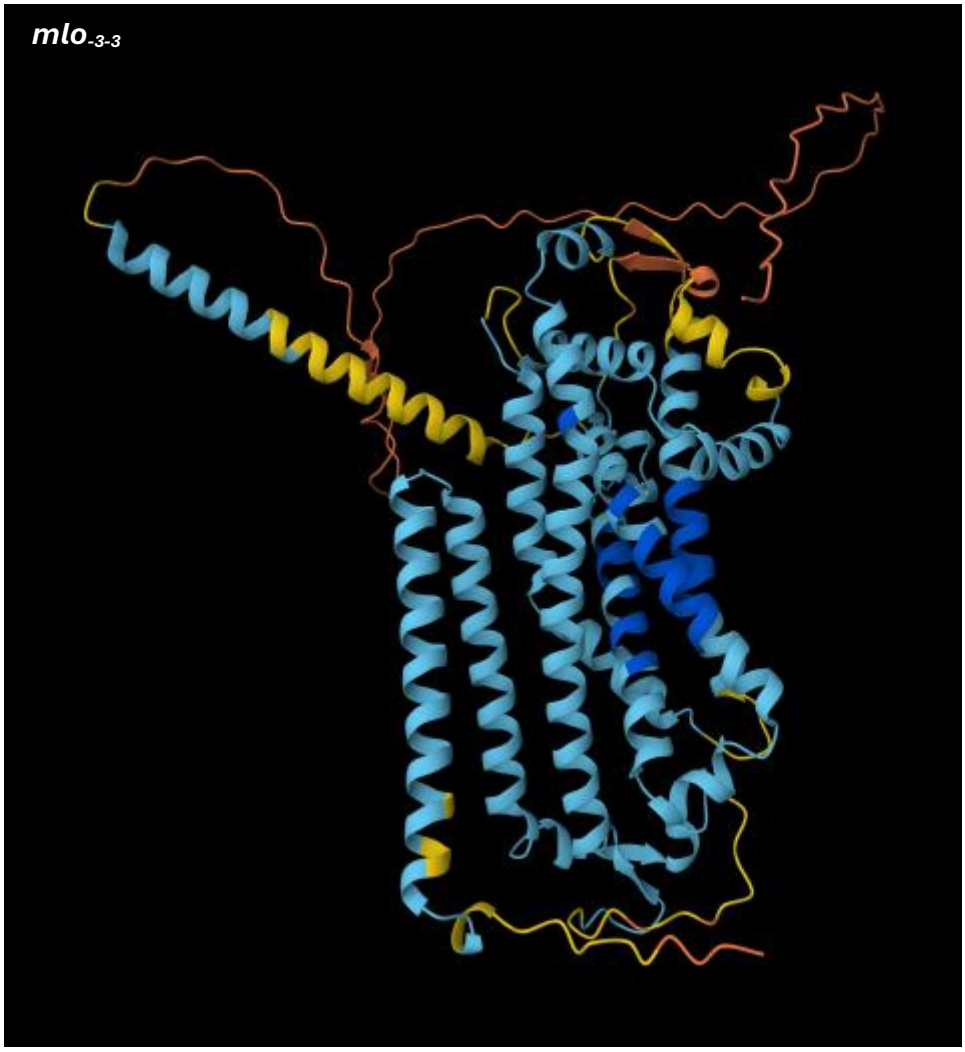
Protein prediction alignment of the *mlo* mutants and 3D protein structure modelling

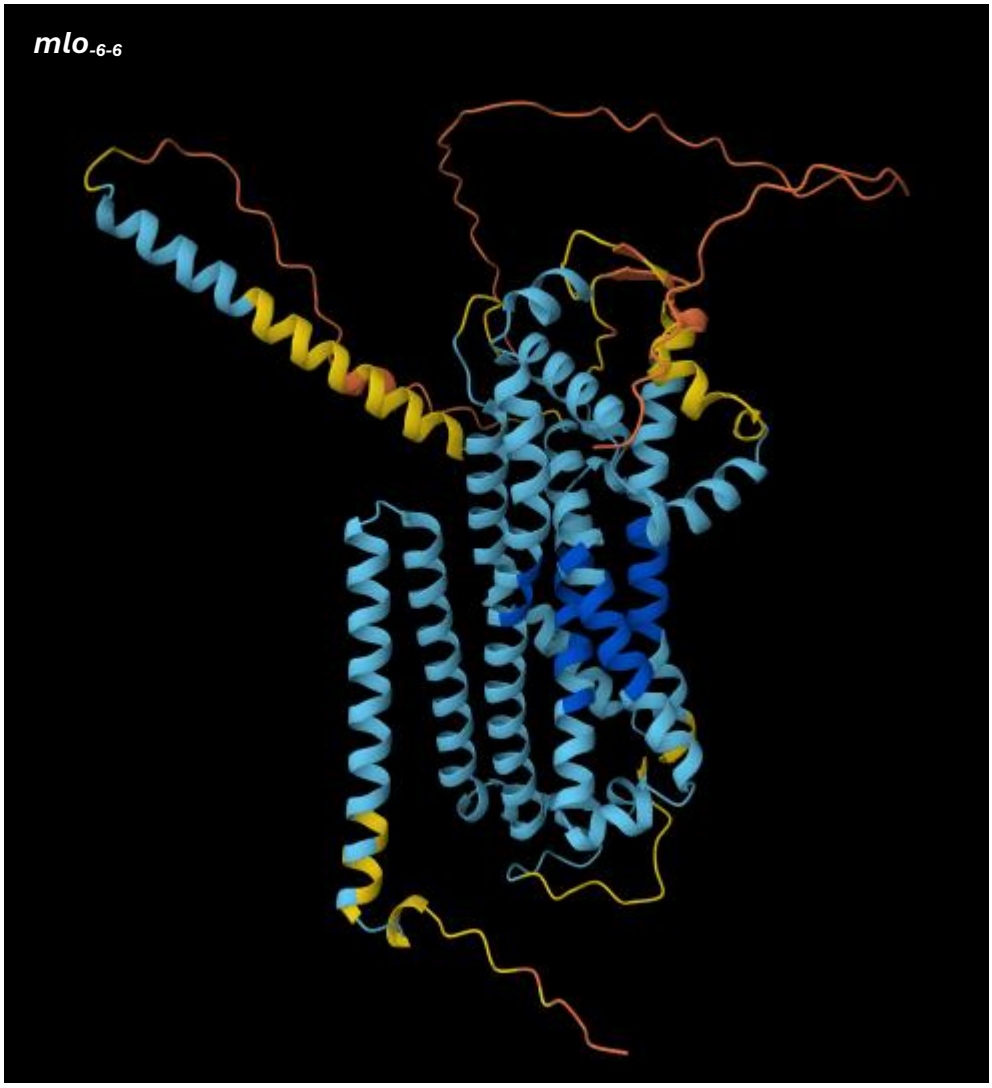
Alignment of the mutants' predicted protein sequences against the wild-type *Mlo* sequence from cv. 'Maythorpe'.

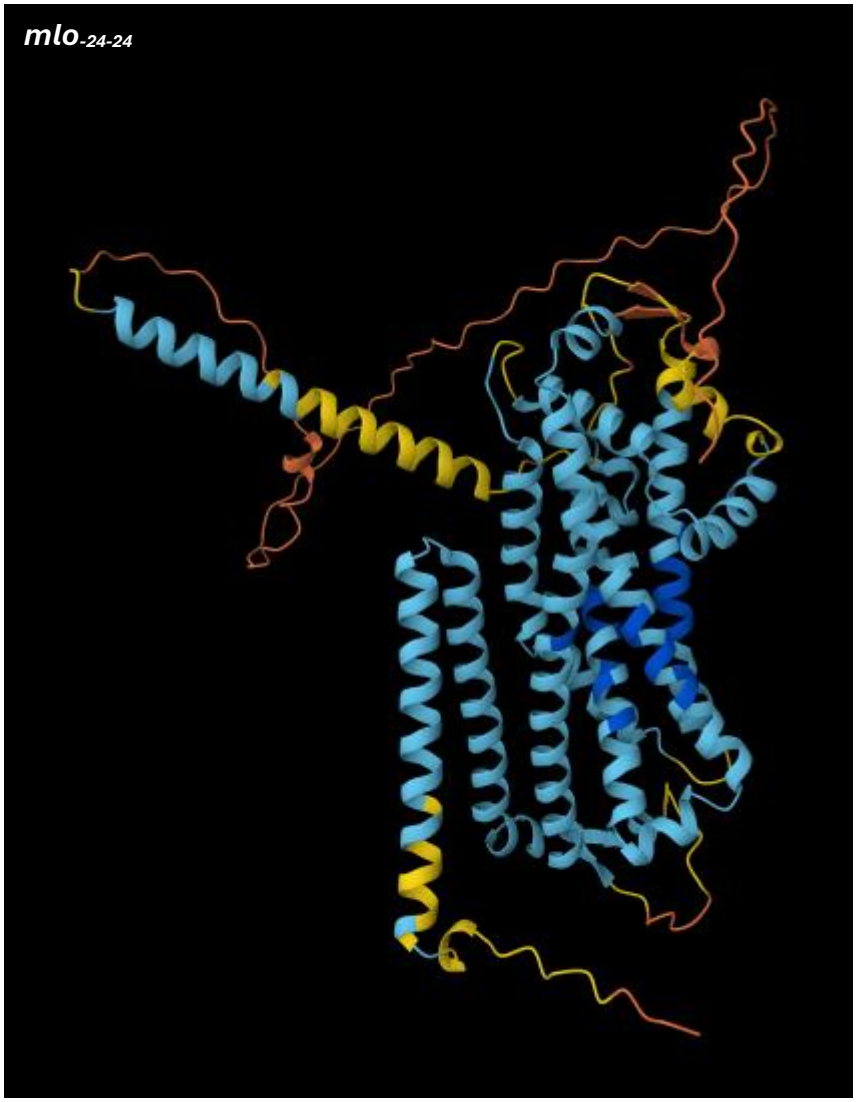


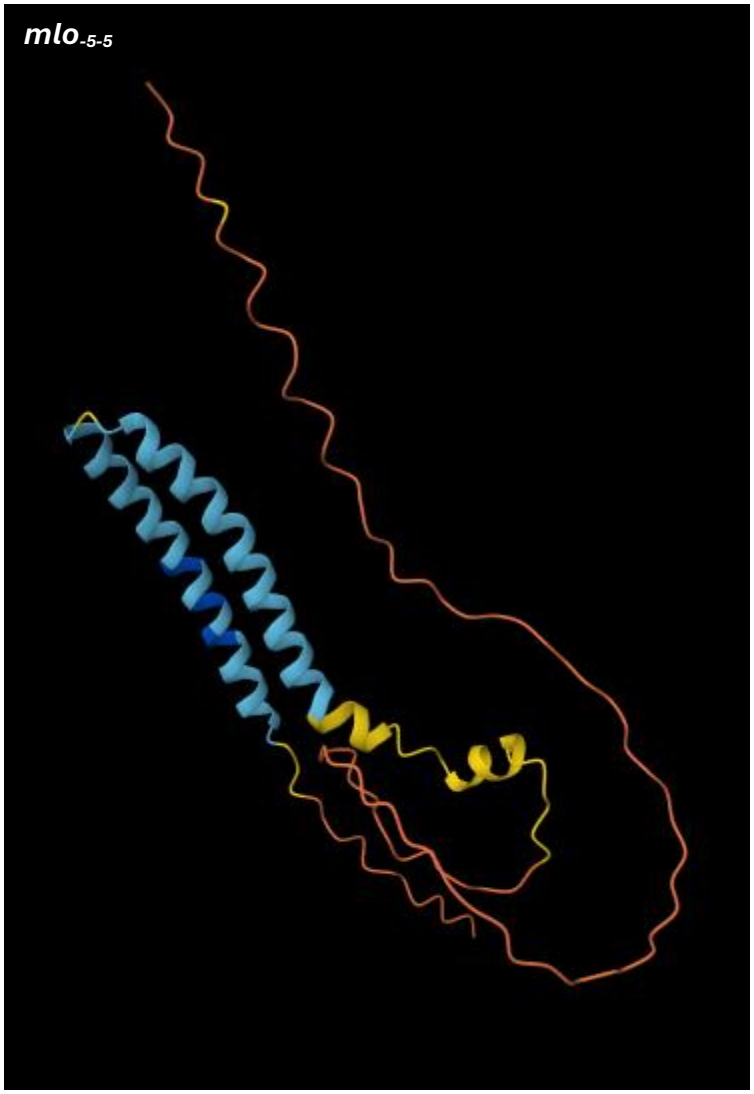
Figure S3. Predicted protein sequences of *mlo* mutants aligned against the wild type sequence. Mismatches are labelled with red.


WT


mlo-3-3


mlo-6-6


mlo-24-24


mlo-5-5

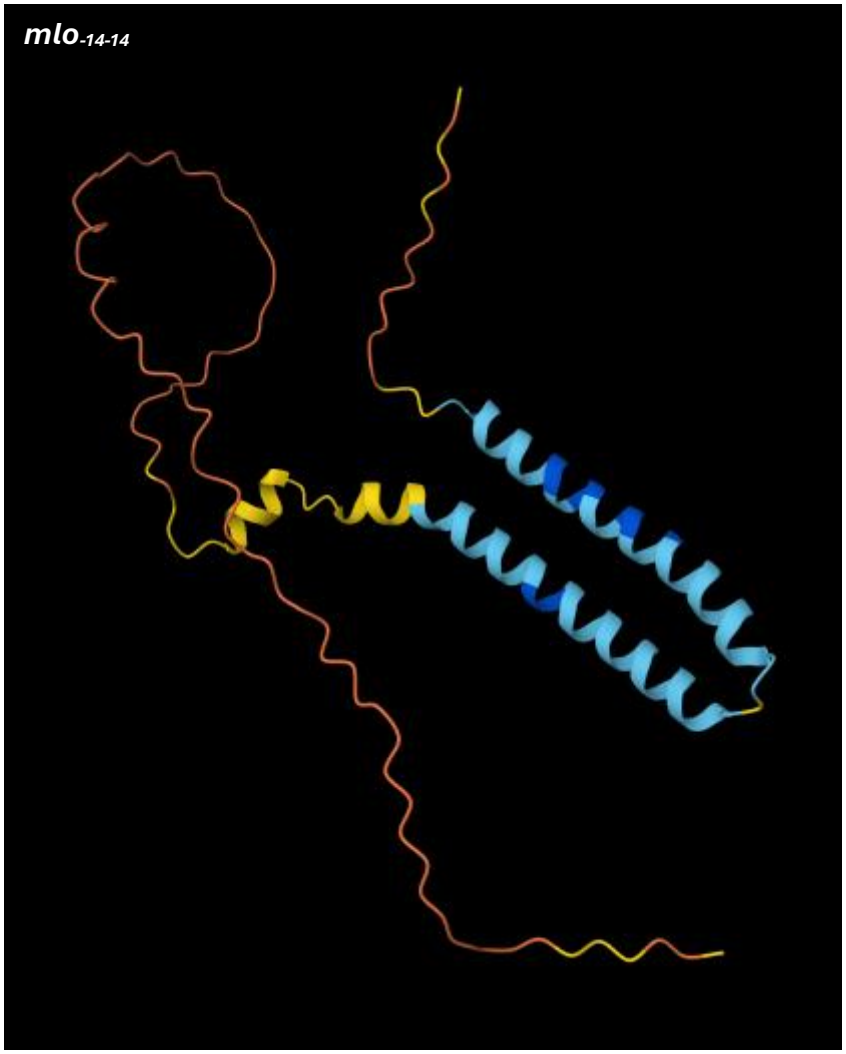


Figure S4. Three-dimensional protein structure models of *mlo* mutants and the wild-type cultivar ‘Maythorpe’.

Setup for inoculation with *Blumeria hordei (Bh)*

This is a visual representation of our setup used for *Bh* inoculation. The tray was placed inside the settling tower, and conidiospores were blown into the tower from a few infected barley plants.

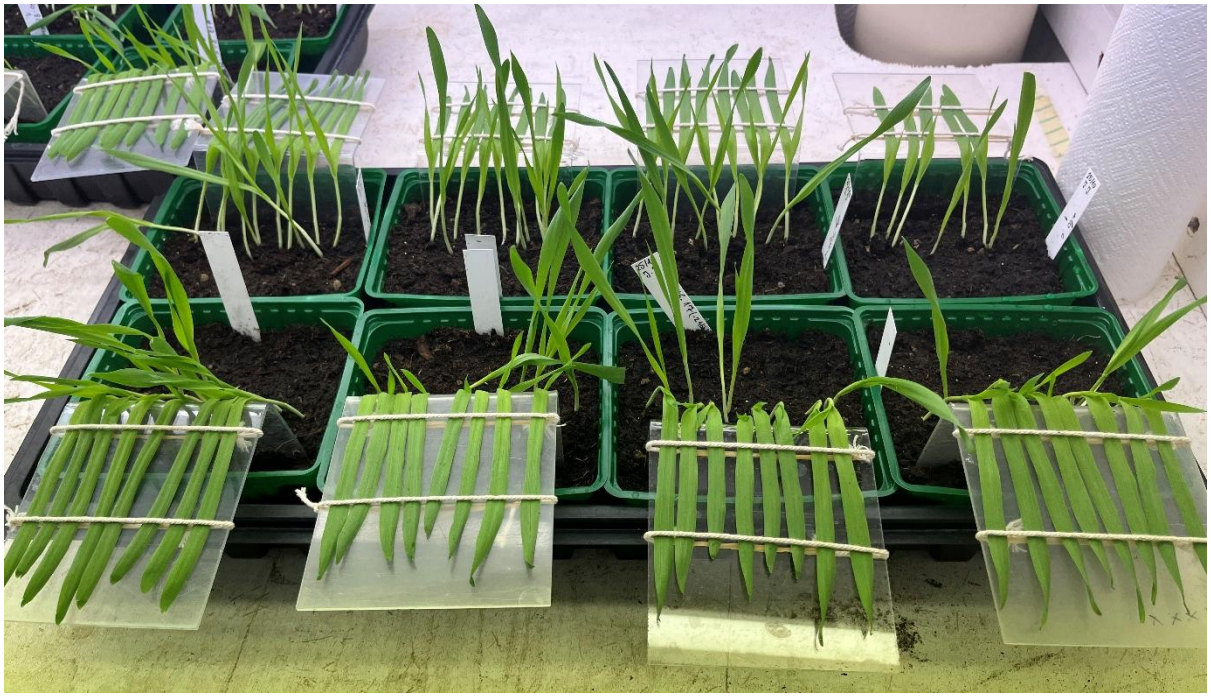


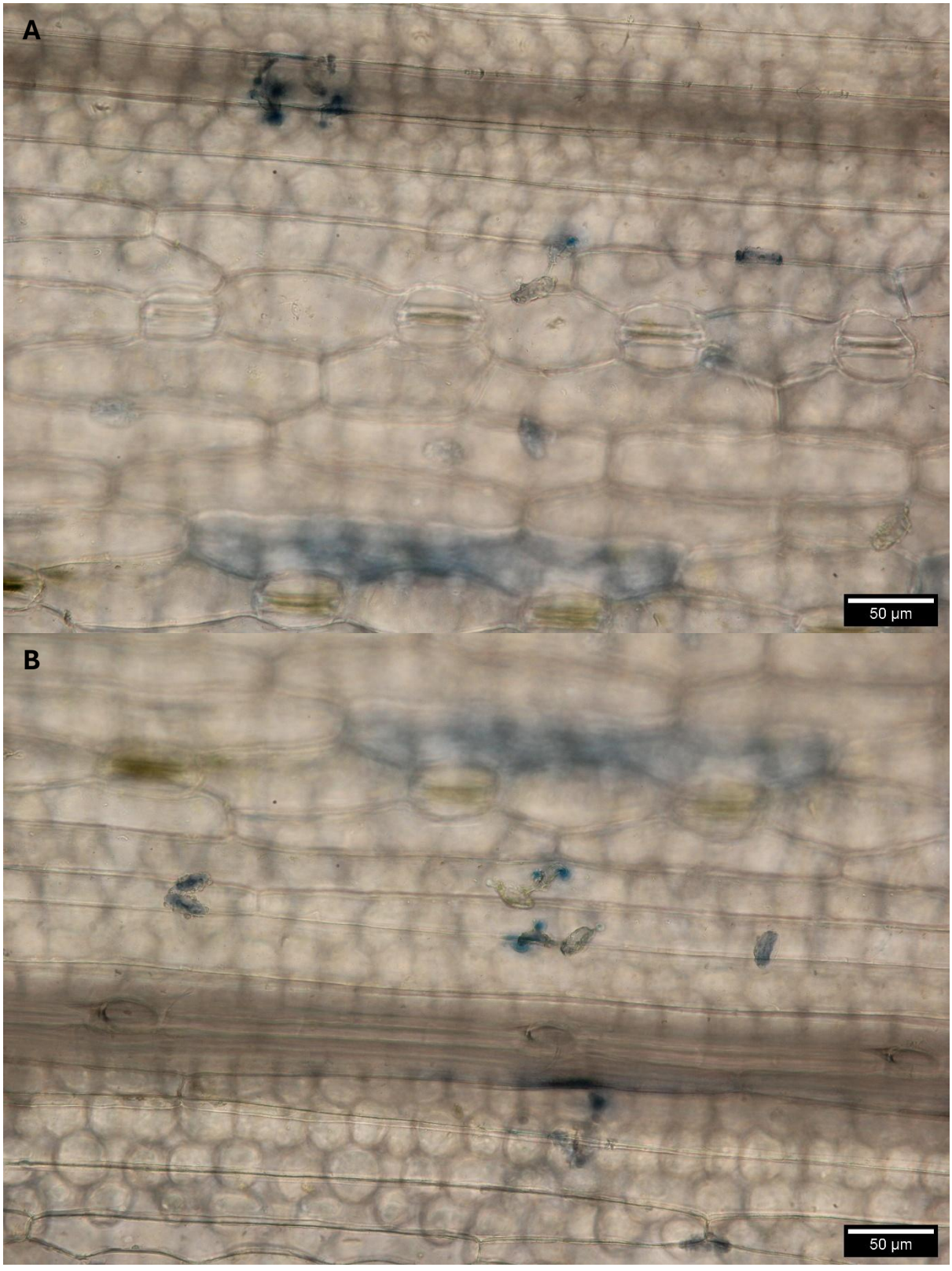
Figure S5. Set up for barley *mlo* mutants' inoculation with *Bh*. Primary leaves were bent over the horizontal plastic support, with adaxial side up, and secured with two bands.

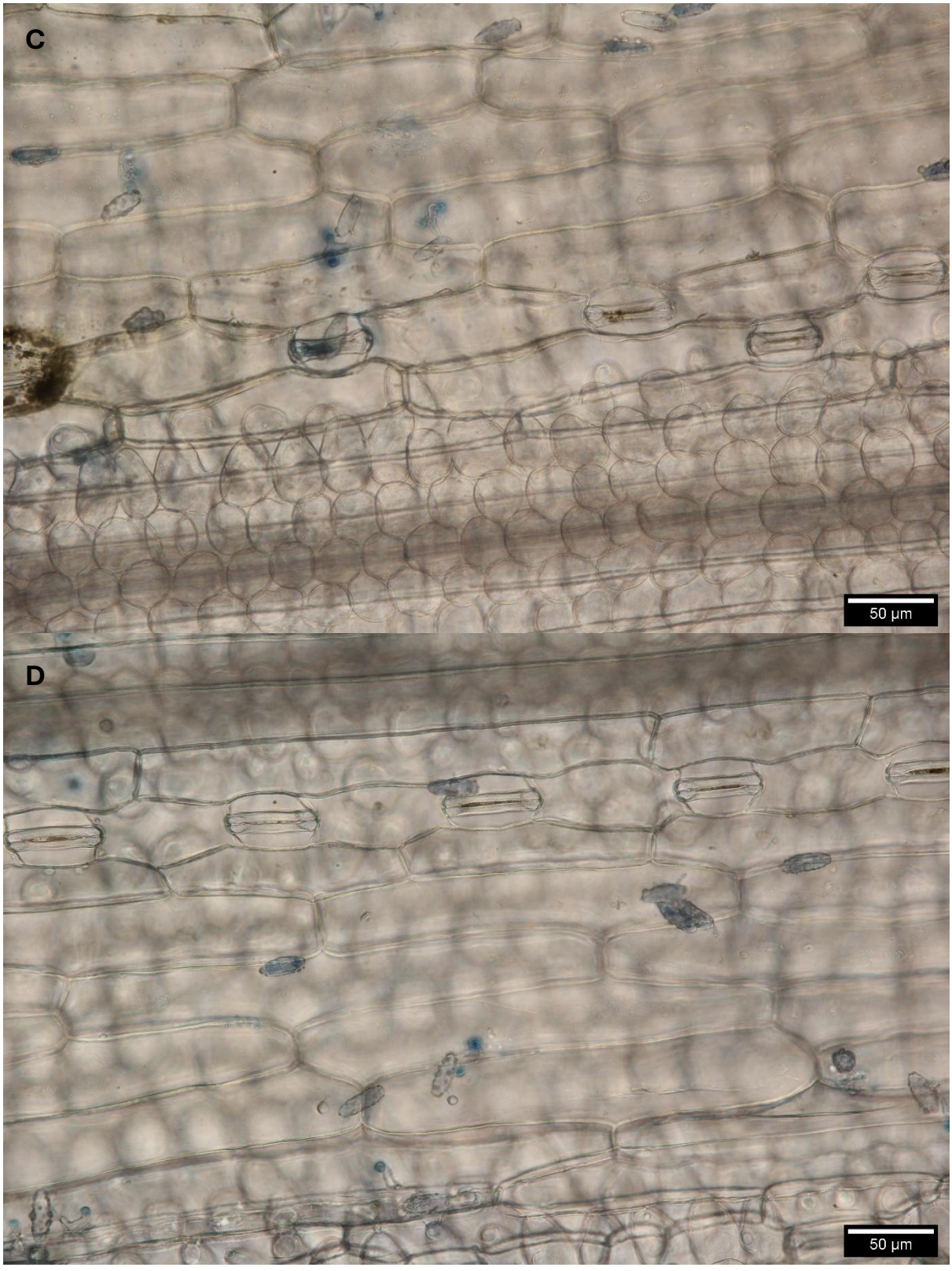
Microscopic resistance scoring

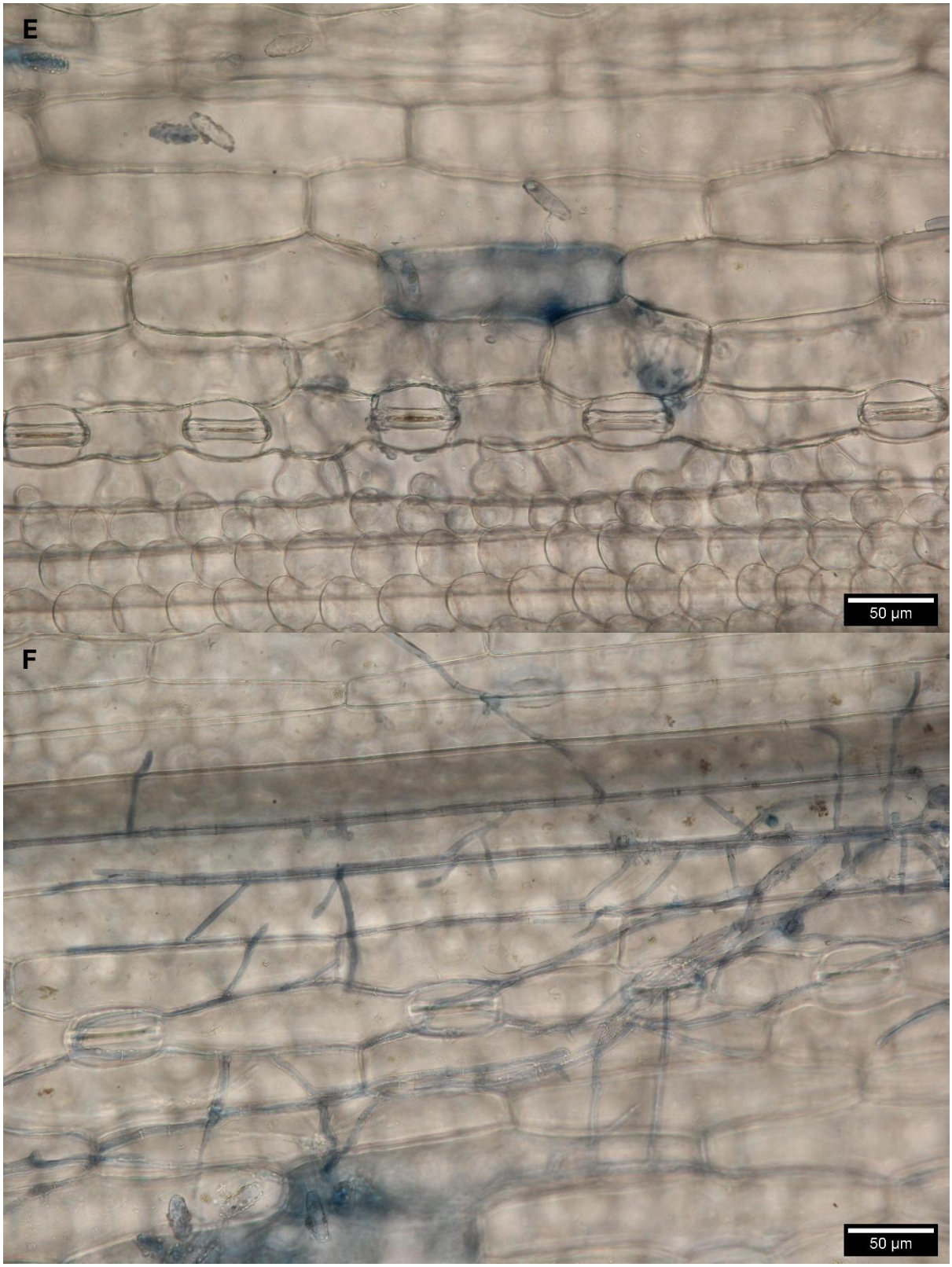
Table S1. Summary of results from the microscopic resistance screening. One hundred penetration attempts were counted per replicate. The table shows the numbers of non-penetration events, hypersensitive responses (HRs), and formed colonies per replicate. Results for all replicates are presented.

Genotype	No penetration	HR	Colony
WT	33	10	57
WT	44	11	45
WT	36	8	56
WT	37	7	56
***mlo*-3-3**	70	23	7
***mlo*-3-3**	77	19	4
***mlo*-3-3**	80	16	4
***mlo*-3-3**	77	18	5
***mlo*-5-5**	78	21	1
***mlo*-5-5**	72	27	1
***mlo*-5-5**	80	20	0
***mlo*-5-5**	80	20	0
***mlo*-24-24**	74	25	1

mlo-24-24	73	27	0
mlo-24-24	66	32	2
mlo-24-24	72	24	4
mlo-14-14	80	17	3
mlo-14-14	80	18	2
mlo-14-14	82	16	2
mlo-14-14	84	13	3
mlo-6-6	61	35	4
mlo-6-6	69	28	3
mlo-6-6	71	25	4
mlo-6-6	69	30	1


A
50 µm
B
50 µm


C
50 µm
D
50 µm


E
50 µm
F
50 µm

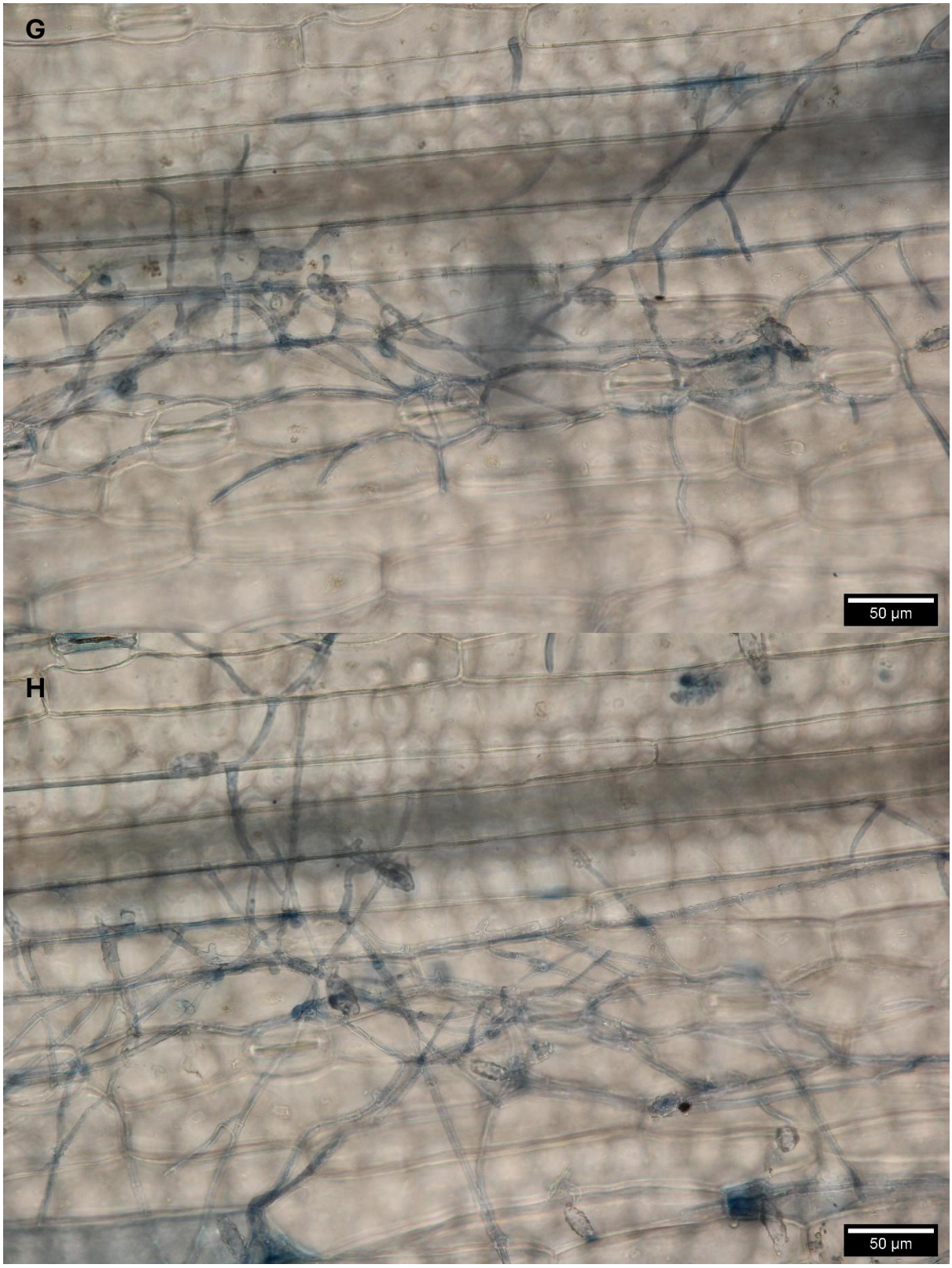


Figure S6. Additional microscopic images of mutants (A–E) and the wild type (F–H) at 4 dpi.