

# Characterization of Nep-like proteins (NLPs) of *Verticillium longisporum* according to their relevance for pathogenicity in *Brassica napus*

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Recently, a new family of small phytotoxic peptides, called Necrosis and Ethylen-inducing Peptides (NEPs), was described. Since the first NEP were detected more than 10 years ago, many other NEP-like proteins (NLPs) were detected in various microbiological species including Bacteria, Oomycetes and Fungi. Most of them are plant pathogens. In this study we identified and characterised five NLP genes in *V. longisporum*, designated as VI-NEP-1 to 5, by molecular techniques. Typical symptoms of stunting and early flowering caused by *V. longisporum* have not been described to be emerged by NLPs since this time. Our findings strongly suggest VI-NEP-1 being a true virulence factor of *V. longisporum* during infection of *B. napus*.

**1. *Verticillium longisporum*** is a soilborne plant pathogen which infects *Brassica* and other cruciferous host plants via the roots and colonizes the vascular system. The most distinctive features of *V. longisporum* infection as compared to other plant pathogenic *Verticillium* species are the limitation of hosts to crucifers and the lack of typical wilt symptoms. The pathogen instead causes stunting, chlorosis and anthocyanin accumulation, affects the flowering time and triggers early onset of senescence.

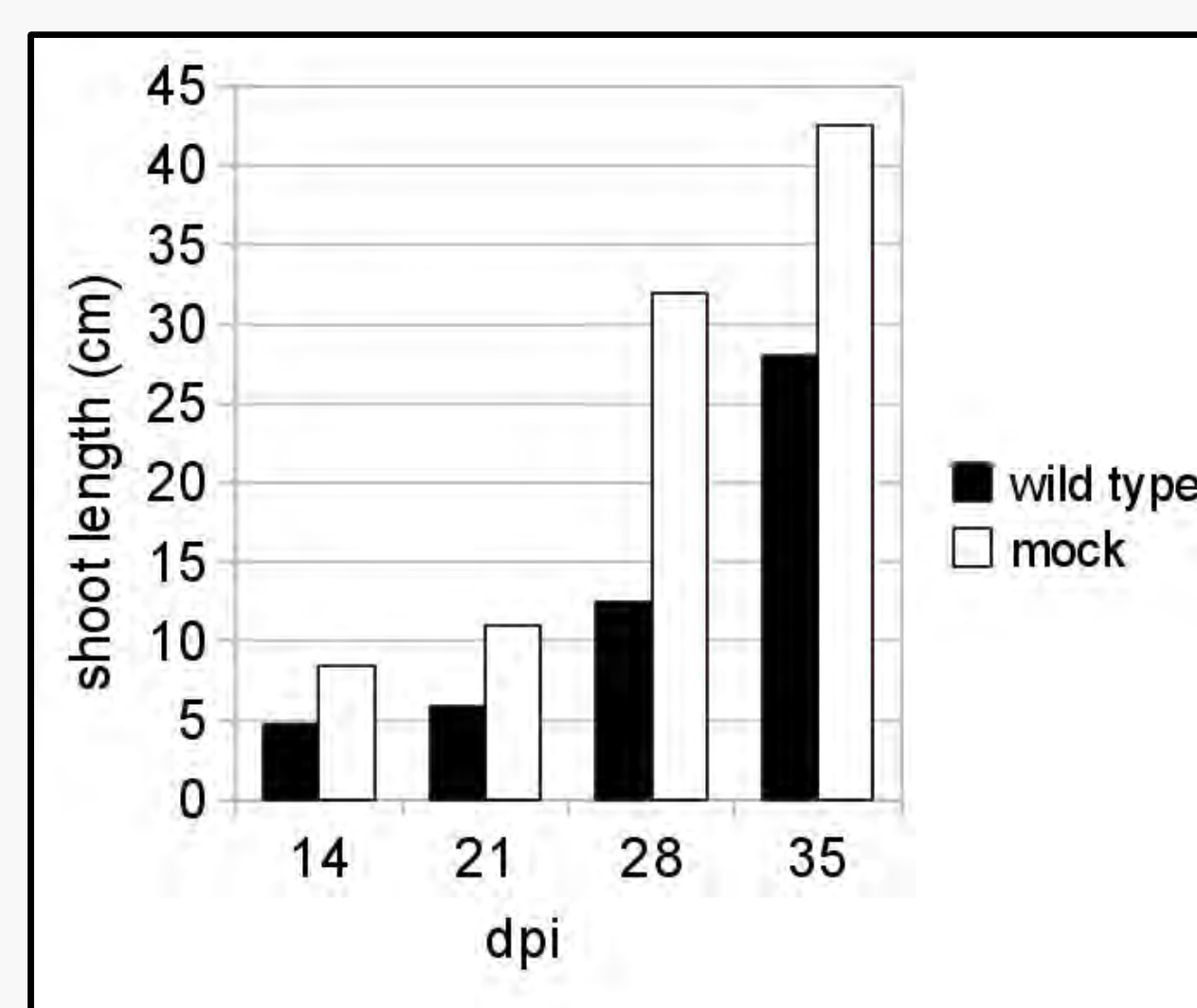


Figure 1. Shoot lengths of *V. longisporum* rape plants in contrast to mock inoculated

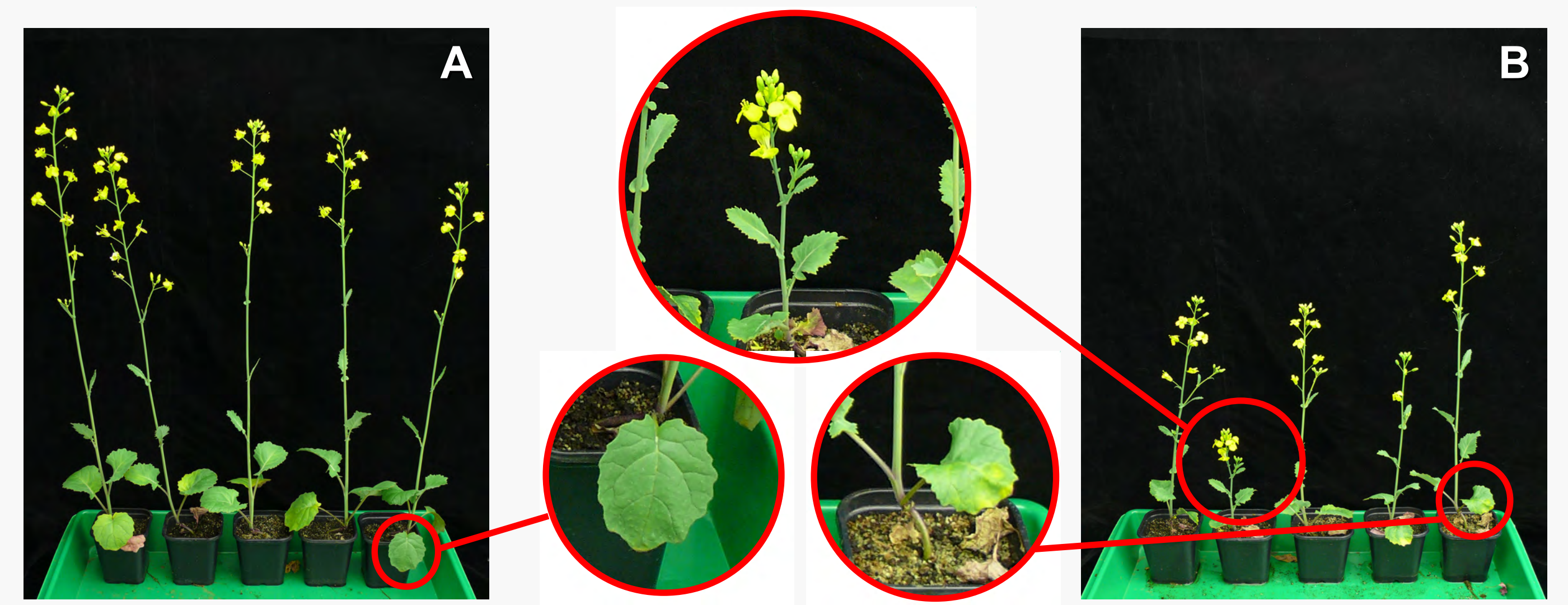


Figure 2. Effects of *V. longisporum* while infecting *B. napus* (stunting and chlorosis) A. Mock inoculated (28 dpi) B. Wild type inoculated (28 dpi)

**2. NEP-like proteins** are widely distributed among microbes described to be excreted by diverse pathogens and to induce ethylene production and necrosis in plants. All members of this family isolated range from 24-36 kDa and comprise a highly conserved region, namely a GHRHDWE motif in the centre. We inspected the *V. dahliae* genome for NLP genes considering molecular weight and the conserved motif and identified 8 distinctive sequences of hypothesized ORFs. We succeeded in PCR-amplified products with genomic DNA of *V. longisporum* either directly or by use of a genome library of *V. longisporum*. All amplicons were sequenced to proof homology to corresponding Vd-NEP genes and specific primers for VI-NEP genes were designed. Sequence properties of VI-NEP genes identified are given in Table 1.

VINEP	ORF (nt)	aa sequence	Mol. weight (Da)	intron	aa sequence identity (Vd)	aa sequence identity (Vaa)
1	760	233	25,986	207-267	229/233 (98%)	224/233 (96%)
2	831	256	27,361	321-380	251/256 (98%)	234/267 (88%)
3	834	277	31,040	-	262/283 (93%)	269/277 (97%)
4	927	308	35,327	-	292/308 (95%)	283/296 (96%)
5	970	264	29,420	92-149 577-631 829-890	264/264 (100%)	185/295 (63%)

Table 1. Sequence properties of 5 VI-NEP genes found in *V. longisporum*

**3. Expression of VI-NEP genes** were measured by qRT-PCR using RNA from *B. napus* hypocotyl tissue of *V. longisporum*-infected plants in contrast to *in vitro* conditions. Strongly enhanced transcript levels of VI-NEP-1, -3 and -5 reach the conclusion that downregulation of these genes could give a closer look into their role during pathogenicity of *V. longisporum*.

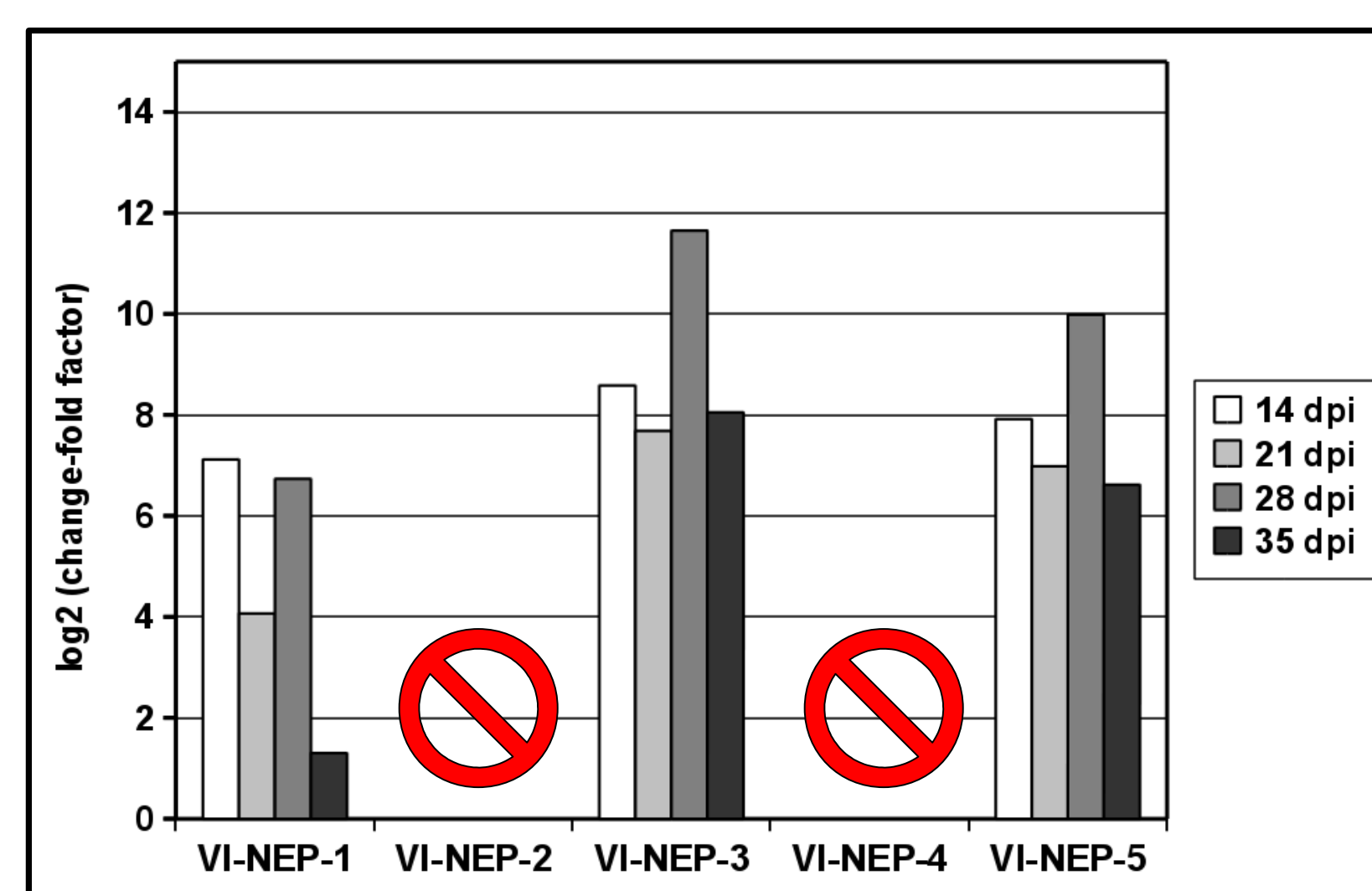


Figure 3. *in planta* Expression of VI-NEP genes in contrast to *in vitro* expression (no expression for VI-NEP-2 and VI-NEP-4 was measured in planta)

**4. For gene silencing** a sequence-specific double stranded hairpin-cassette for VI-NEP-1 was constructed with molecular techniques. The fungus was transformed by *Agrobacterium thumefaciens* with the pPK2-VI-NEP-1 vector for over-expression of an VI-NEP-1 silencing cassette to trigger RNAi. Seven positive transformants were identified on hygromycin-selective medium

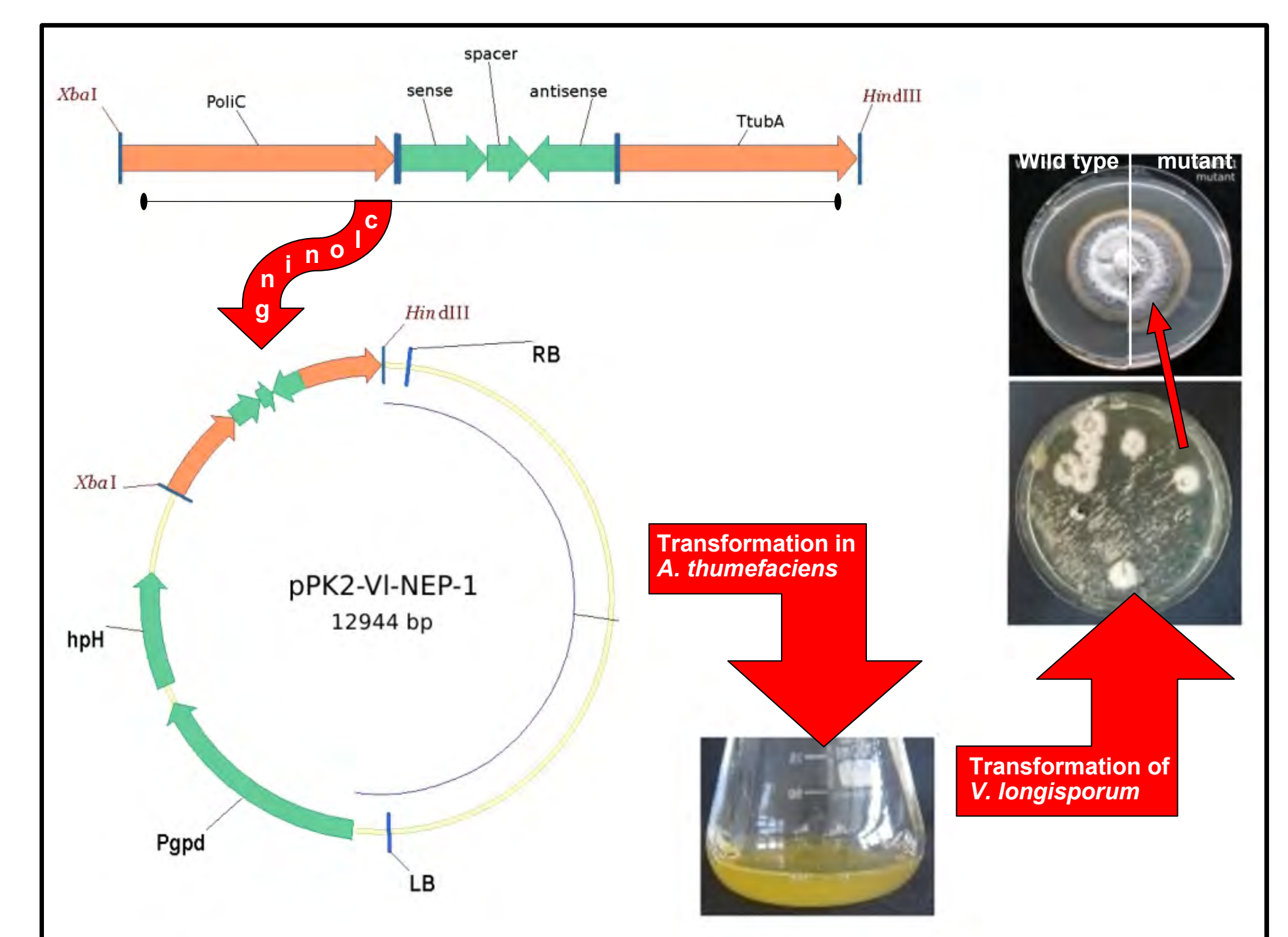


Figure 4. Hairpin-construct for post-transcriptional inhibition of VI-NEP-1 and transformation into *V. longisporum*

**5. VI-NEP-1 transformants** were tested by PCR and also by Southern hybridisation. An additional third signal on the blot for the VI-NEP-1 mutants verifies the successful transformation of *V. longisporum* with the silencing cassette for the post-transcriptional suppression of VI-NEP-1.

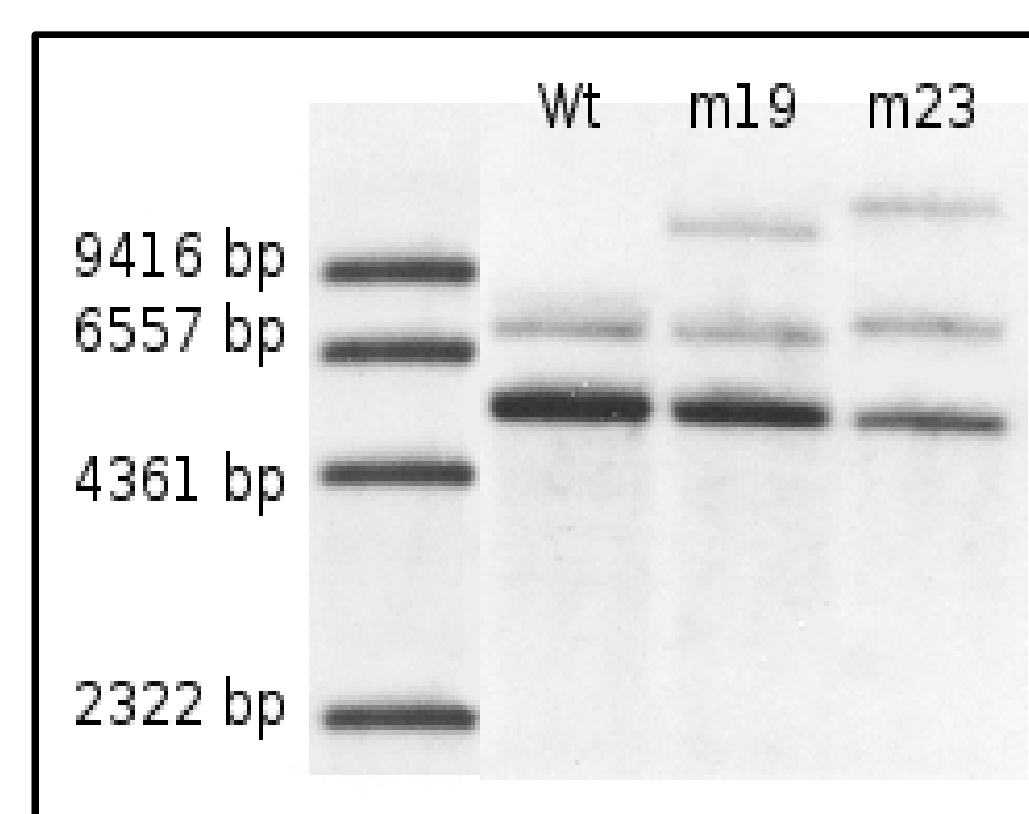


Figure 5. Southern blot of VI-NEP-1 mutants

**6. Silencing efficiency** was measured by qRT-PCR. For this, we extracted RNA from mycelium of 7 VI-NEP-1 mutants and measured the expression in comparison to wild type. Five VI-NEP-1 mutants show a higher silencing efficiency than 80%.

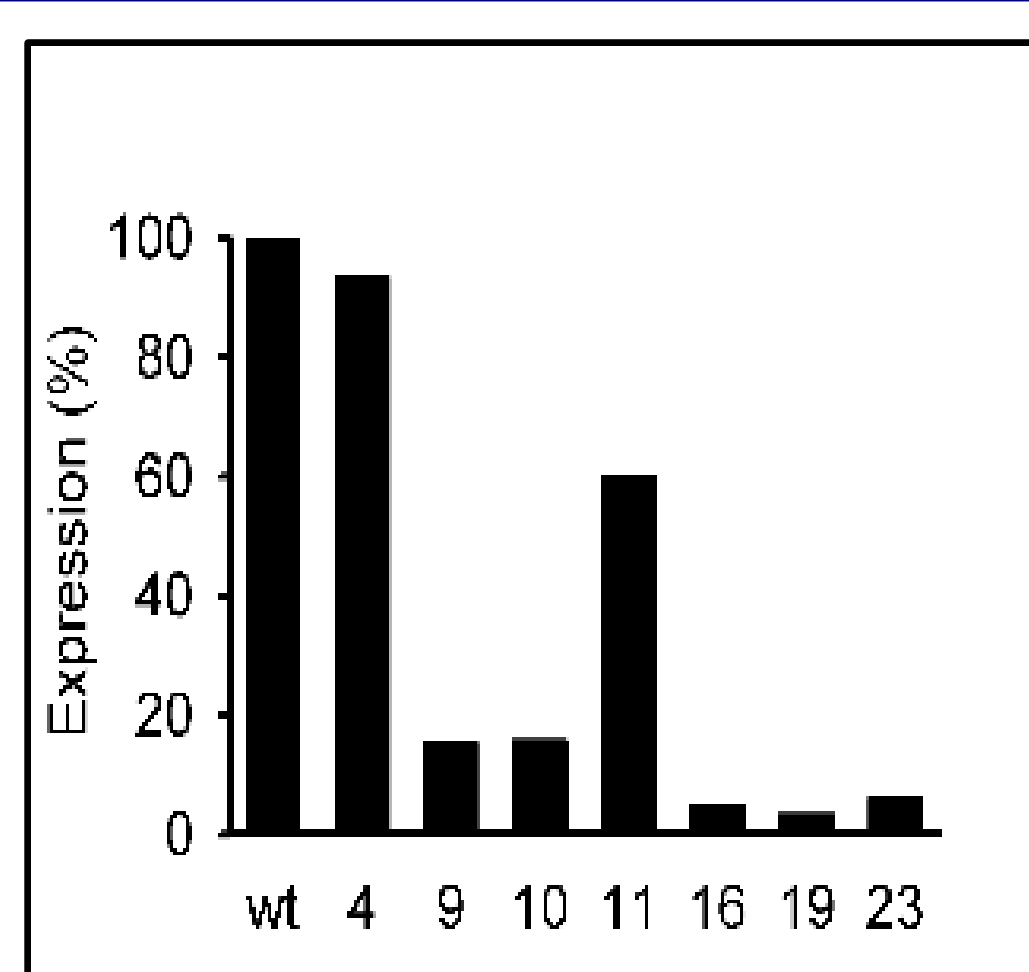


Figure 6. Silencing efficiency of VI-NEP-1 mutants

**7. Pathogenicity assay on *B. napus*** was done to investigate the significance of VI-NEP-1 during the pathogenesis and symptom development of *V. longisporum*. For this we infected rape plants with *V. longisporum* wild type and two VI-NEP-1 knock-down mutants (m19 and m23). Symptoms were evaluated by measuring the plant shoot lengths and by assessment of a disease score. After 14 dpi a reduction in symptom severity were observed which became significant at 21 dpi and 28 dpi in VI-NEP-1 transformants compared to wild-type showing less stunting in infected *Brassica* plants. These results suggest that VI-NEP-1 is a true virulence factor of *V. longisporum* while infecting *B. napus*.

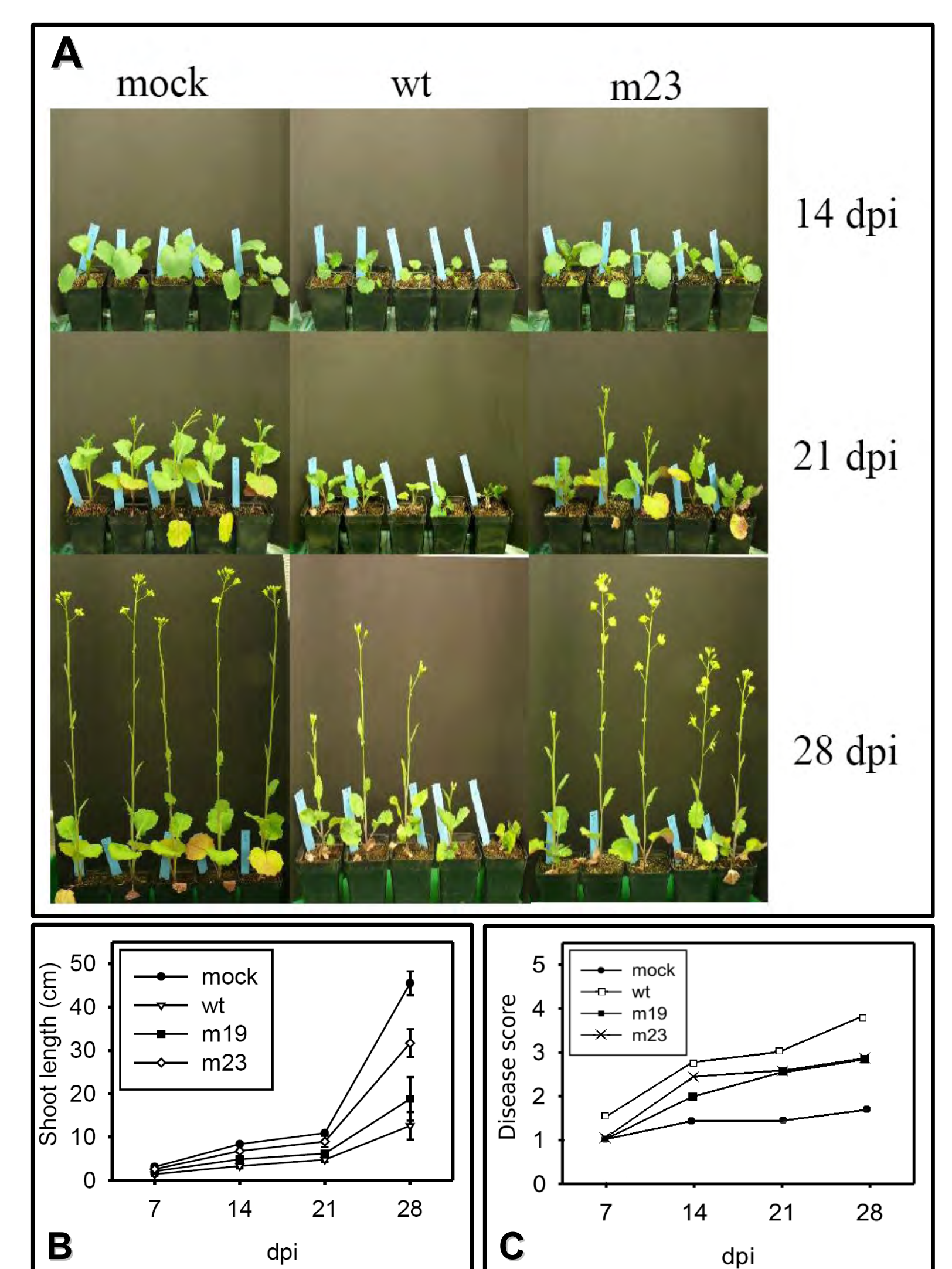


Figure 7. A. Pathogenicity assay on *B. napus* B. Shoot length of infected plants C. Disease score of infected plants