BRIEF COMMUNICATION

BNYVV-derived dsRNA confers resistance to rhizomania disease of sugar beet as evidenced by a novel transgenic hairy root approach

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Abstract *Agrobacterium rhizogenes*-transformed sugar beet hairy roots, expressing dsRNA from the *Beet necrotic yellow vein virus* replicase gene, were used as a novel approach to assess the efficacy of three intron-hairpin constructs at conferring resistance to rhizomania disease. Genetically engineered roots were similar in morphology to wild type roots but were characterized by a profound abundancy, rapid growth rate and, in some cases, plagiotropic development. Upon challenge inoculation, seedlings showed a considerable delay in symptom development compared to untransformed or vector-transformed seedlings, expressing dsRNA from an unrelated source. The transgenic root system of almost all seedlings contained no or very low virus titer while the non-transformed aerial parts of the same plants were found infected, leading to the conclusion that the hairy roots studied were effectively protected against the virus. This readily applicable novel method forms a plausible approach to preliminarily evaluate transgenic rhizomania resistance before proceeding in transformation and whole plant regeneration of sugar beet, a tedious and time consuming process for such a recalcitrant crop species.

Keywords *Beta vulgaris* · Beet necrotic yellow vein virus · *Agrobacterium rhizogenes* · Transformation · Sugar beet · Rhizomania

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Introduction

Rhizomania disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (Tamada and Baba 1973), which is vectored and transferred to sugar beet healthy roots by the widespread soilborne plasmodiophorid fungus *Polymyxa betae* Keskin. Due to a dramatic reduction in root yield, sugar content and purity when susceptible cultivars are grown, the potential sugar yield losses may reach up to 90% (Johansson 1985). Consequently, the economic viability of the crop is largely dependent on the successful protection against rhizomania. The only practical means of combating this most devastating disease is the use of genetically resistant varieties and therefore, all relevant breeding activities become of paramount importance. All cultivars in commercial use have been developed through

conventional breeding methods, including marker-assisted backcross breeding approaches (for a review, see De Biaggi 2005). Despite breeding progress, a better yield is needed under disease conditions and, various genetic engineering approaches are also being employed to further improve disease resistance (for a review, see Skaracis 2005). Among these, RNA silencing-mediated resistance has recently attracted special interest (Andika et al. 2005; Lennefors et al. 2006).

RNA silencing is a highly conserved mechanism that occurs in a wide range of eukaryotic organisms, including animals (RNA interference, RNAi), plants (post-transcriptional gene silencing, PTGS) and fungi (quelling), and functions as an innate defense system against alien nucleic acid molecules (Voinnet 2001; Tijsterman et al. 2002). The mechanism is a RNA-based process acting in a sequence-specific manner to control gene expression either at a transcriptional or post-transcriptional level (Baulcombe 1999; Broder-sen and Voinnet 2006). A key feature of RNA silencing refers to its role as an active defense system against viral invaders in plants and invertebrates (Ding and Voinnet 2007) and against transposable elements in mammals (Tabara et al. 1999; Kalmykova et al. 2005; Vagin et al. 2006). The triggering element of all existing RNA silencing pathways is a dsRNA mole-cule, capable of recruiting necessary components to carry out sequential phases of sequence-specific degradation (Fire et al. 1998; Hammond et al. 2001). Following recognition, dsRNA is cleaved by the nuclease DICER, a member of an RNase III-like enzyme family, and processed into short, 21–23 nucleotides in length, interfering RNAs with 2nt-overhangs at the 3' ends (siRNAs) (Hamilton and Baulcombe 1999; Bernstein et al. 2001). After strand separation, unwound ssRNAs are incorporated into the RNA-induced silencing complex (RISC) and serve as guides for recognition of homologous RNAs in the cytoplasm, leading to inhibition of transcript stability or translation (Hammond et al. 2000; Song et al. 2004; Hammond 2005).

Since its discovery, RNA silencing (Napoli et al. 1990; van der Krol et al. 1990) has been used in gene knock down studies in a variety of eukaryotic organisms (Fire et al. 1998; Ngo et al. 1998; Lohmann et al. 1999; Smith et al. 2000). In plants, RNA silencing primarily aims at the development of antiviral activity (Voinnet 2001, 2008; Waterhouse et al. 2001; Vazquez

et al. 2002; Goldbach et al. 2003; Tenllado et al. 2004), but is also employed in transposon silencing, tran-scriptional gene silencing due to DNA methylation, chromatin condensation and as a tool for gene regulation studies (Stam et al. 1997; Hamilton et al. 2002; Brodersen and Voinnet 2006). The mechanism is usually triggered by the introgression of transgene constructs, arranged as inverted repeats ("hairpin" molecules), capable of duplex RNA formation (Ham-ilton et al. 1998; Waterhouse et al. 1998; Chuang and Meyerowitz 2000; Johansen and Carrington 2001).

The aim of this work was to study three different hairpin constructs, carrying parts of a highly conserved region from the replicase gene of BNYVV, in confer-ring transgenic resistance to the rhizomania disease. As is well known, sugar beet is particularly recalcitrant to transformation species requiring a tedious, time consuming and costly procedure to obtain a satisfac-tory number of successfully transformed and regener-ated plants (D' Halluin et al. 1992; Krens et al. 1996; Wozniak 1999). In the light of such difficulties, we have developed a novel method for *A. rhizogenes*-mediated production of plants with a transgenic hairy root system and non-transformed shoots and leaves to allow for the evaluation of transgene effect on virus accumulation. Such a shortcut approach can be easily employed and would be of significant value in providing relevant information prior to effecting the laborious and problematic transformation and whole plant regeneration.

Materials and methods

Construction of plant expression vectors

Three intron-hairpin constructs from the BNYVV replicase gene, encoded by RNA1, were assembled and used for *A. rhizogenes*-mediated transformation. Total RNA extracted from BNYVV-infected sugar beet roots was reversed transcribed using gene-specific primers and the Im-Prom II Reverse Trans-criptase System (Promega) and used as template for PCR to obtain 459, 589 and 824 bp fragments with primer pairs GWAttb$_1$1F-GWAttb$_2$1R, GWAttb$_1$2F-GWAttb$_2$1R and GWAttb$_1$2F-GWAttb$_2$2R, respec-tively (Table 1). PCR reaction mixture contained 2.5 µl cDNA, 0.5 µM of each primer, 200 µM dNTPs, 1× HF buffer and 1 u of high fidelity DNA

Table 1 Primers used to obtain the BNYVV replicase-derived transgene sequences

Primer	Sequence (5' → 3')	nt position	Product size (bp)
GWAttb$_1$ 1F	GGGGACAAGTTTGTACAAAAAAGCAGGCT TTTCTGACTTCTTTTTGGATTG	RNA 1: 5750	
			824 (GW-IR2)
GWAttb$_2$ 1R	GGGGACCACTTTGTACAAGAAAGCTGGGT TCAAGATAGGAGGCCTGTGGCAT	RNA 1: 6574	
			459 (GW-IR3)
GWAttb$_1$ 2F	GGGGACAAGTTTGTACAAAAAAGCAGGCT CGAAGATAGCAGCACACAGGTTC	RNA 1: 6116	
			589 (GW-IR1)
GWAttb$_2$ 2R	GGGGACCACTTTGTACAAGAAAGCTGGGT TTCACACCCAGTCAGTACA	RNA 1: 6704	

polymerase (Phusion High Fidelity DNA polymerase, Finnzymes) in a volume of 50 μl. Amplification cycles included a first denaturation cycle of 30 s at 98°C, then 35 cycles composed of 10 s at 98°C, 30 s at 60°C, 45 s at 72°C with a final elongation cycle of 10 min at 72°C. PCR-amplified products were subsequently eluted from agarose gels and used for the development of recombinant plasmids using the Gateway Cloning Technology (Invitrogen), according to the instruction manual. Amplicons corresponding to BNYVV replicase gene were initially introduced by a recombination reaction using Gateway BP clonase enzyme mix to pDONR 221, leading to the creation of entry clones. Transgenes were subsequently recombined with the destination vector pK7WIWG2 (II) (Karimi et al. 2002) carrying neomycin phosphotransferase II (*npt*II) as a selectable marker gene, using Gateway LR clonase enzyme mix, to generate the binary plant expression vectors GW-IR1, GW-IR2 and GW-IR3. Following restriction digestion, for the confirmation of integrity and orientation of the hairpin structure, plasmid DNA was rescued from transformed *E. coli* cells and sequenced using M13 forward and reverse primers.

Bacterial strains

The *A. rhizogenes* strain R1000 harbouring the plasmid pRiA4 was used to transform sugar beet seedlings. The binary plant expression vectors GW-IR1, GW-IR2, GW-IR3 and the destination vector pK7WIWG2 (II), carrying the *ccdB* gene, were introduced to *A. rhizogenes* cells by electroporation and grown at 28°C under spectinomycin (50 μg ml^{-1}) and nalidixic

acid (25 μg ml^{-1}) selection for 2 days or until OD$_{600}$ = 0.6–1.0 was reached. Bacterial cells were collected by centrifugation for 10 min at 1,500 rpm and the pellet was used as inoculum for plant transformation.

Plant material

Transformation experiments were carried out using the commercial sugar beet variety Alexandra, which is a diploid, monogerm, three-way top cross hybrid (EU Common Variety Catalogue, ed. 27, E297A/20-11-2008). Alexandra is a standard cercospora leaf spot resistant-rhizomania susceptible variety.

A. rhizogenes-mediated transformation and evaluation of transformed roots

Transformation was performed on 9 day-old aseptically grown sugar beet seedlings by removing the existing root system and dipping the wounded surface of hypocotyl into the bacterial cells. Inoculated seedlings were co-cultivated for 3 days (23°C, 16/8 h light/darkness) in square petri dishes containing co-cultivation medium (½ MS basal salts, 1% sucrose, 0.9% PhytoAgar), half-covered with filter paper and subsequently transferred to selection medium (½ MS containing 2-MorpholinoEthaneSulfonic acid (MES) and vitamins (1 μg ml^{-1} glycine, 50 μg ml^{-1} myoinositol, 0.25 μg ml^{-1} nicotinic acid, 0.25 μg ml^{-1} pyridoxine HCl, 0.05 μg ml^{-1} thiamine HCl), 2% sucrose, 0.9% PhytoAgar, cefotaxime (250 μg ml^{-1}) and kanamycin (150 μg ml^{-1})). Petri dishes were partially sealed and placed vertically in a growth
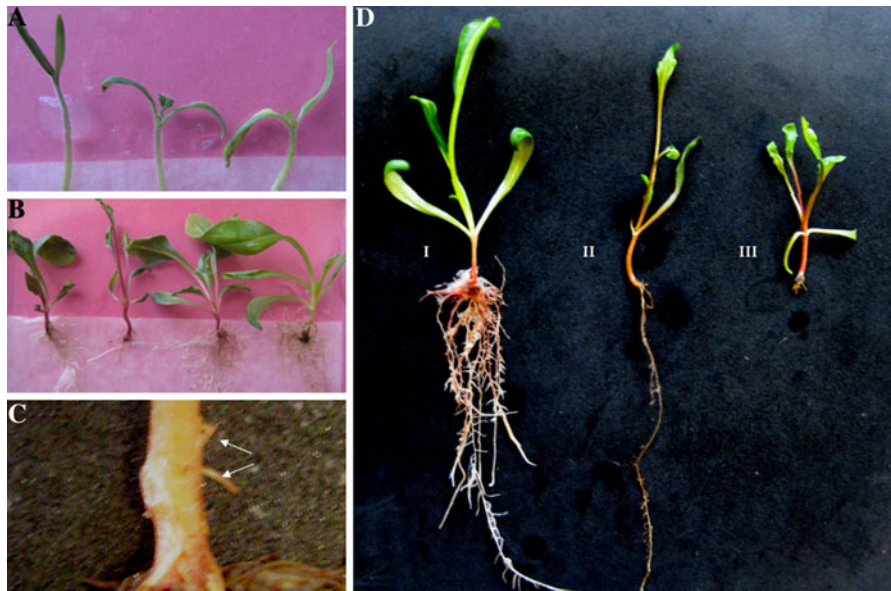
Fig. 1 Production of transgenic sugar beet hairy roots. **a** *A. rhizogenes*-inoculated seedlings (0 dpt) grown vertically on square Petri dishes with a filter paper. **b** Hairy root formation at 10 dpt with *A. rhizogenes* strain R 1000. **c** Emergence of adventitious lateral roots as a response to sectioning. **d** Use of kanamycin for the selection of transgenic hairy roots on composite sugar beet seedlings. *I* Seedling grown on medium containing kanamycin (150 μg ml^{-1}), 10 days after sectioning and inoculation with *A. rhizogenes* strain R 1000. *II* Untransformed seedling under no antibiotic selection. *III* Untransformed seedling grown on kanamycin-containing medium

chamber (Fig. 1a). After growth for 7–10 days, seedlings were regularly monitored for the emergence of new roots and those with well developed hairy roots were transferred and maintained in ½ MS basal salt liquid medium under kanamycin selection. Untransformed sugar beet seedlings obtained by cutting at the hypocotyl, removing the existing root system and grown with or without antibiotic selection, were included as experimental controls.

Kanamycin-resistant sugar beet roots were evaluated for the presence and expression of the cassette and for absence of *A. rhizogenes* cells. Transgene presence and absence of *A. rhizogenes* was examined by a multiplex PCR assay targeting the nucleotide sequences of the BNYVV replicase transgene, the *npt*II gene and *virCD* of *A. rhizogenes*, using FTA (Whatman)-immobilized nucleic acids as template for amplification. Targeted sequences were amplified using the following primer pairs: GWAttb$_1$1F-GWAttb$_2$1R, GWAttb$_1$2F-GWAttb$_2$1R and GWAttb$_1$2F-GWAttb$_2$2R for transgenes, *npt*II F: AGTGA-CAACGTCGAGCACAG-*npt*II R: GCGTTCAAAAGTCGCCTAAG for *npt*II and *virCD* F: CTCATCAGGCACGCTTG-*virCD* R: GCGGATGCTTCAAATGG

for *virCD*. PCR reaction mixture contained template DNA corresponding to 2 mm FTA-saturated with root tissue, 0.25 μM of each primer, 200 μM dNTPs, 1.25 mM MgCl$_2$, 1× *Taq* buffer and 1.25 u *Taq* polymerase (GoTaq Flexi DNA polymerase, Promega) in a final volume of 20 μl. Amplification cycles included a cycle of 3 min at 94°C, then 30 cycles composed of 30 s at 94°C, 1 min at 50°C, 1 min at 72°C with a final cycle of 7 min at 72°C. Amplification products were visualized in agarose gels stained with ethidium bromide. For the evaluation of transgene expression, total RNA was isolated with the SV Total RNA Isolation Kit (Promega) and used as a template in a two step RT-PCR assay targeting the sequences of the inserted transgenes, using the primer pairs and reaction conditions previously described.

Virus inoculations

Heavily BNYVV-infected sugar beet plants were used as virus source for the inoculation of transformed roots. Inoculum was prepared by grinding infected sugar beet root tissue (1:5 w/v) in 0.05 M phosphate buffer containing 0.01% Na$_2$SO$_3$, pH-7.2.

Seedlings were mechanically inoculated by a vortex method adapted from Koenig and Stein (1990). Seedlings were individually placed into test tubes and inoculated by pouring the crude extract (1–1.5 ml per tube) and carborundum as a means to artificially create wounds. Following gentle agitation for at least 3 h, seedlings were subsequently maintained in ½ MS basal salt liquid medium. Vector-transformed seedlings, carrying the *ccdB* gene, and untransformed seedlings served as susceptible controls, whereas untransformed seedlings non-challenged by the virus were included as experimental negative controls.

Assessment of virus resistance

Each seedling was individually analyzed. Seedlings were initially rinsed with tap water to avoid any probable presence of inoculum on the surface of roots and subsequently dried in paper towels. Plant sap from root and leaf tissue was separately extracted in a 1:3 or 1:5 (weight:volume) extraction buffer. Virus titers were measured 14 and 21 days post inoculation (dpi) by DAS-ELISA (Biorad). Each leaf and root sample of the individual seedlings was replicated twice in the plate. Absorbance values at 405 nm measured at least three times the respective measurement of the negative controls were considered as positive.

Data were analyzed, when appropriate, using the statistical package JMP v.6. The differences among root and leaf ELISA readings were statistically compared by a paired *t*-test, based on the respective readings of each individual seedling. ELISA readings on leaves of the four categories tested (three constructs and control) were analyzed by ANOVA and the means were compared by the F protected LSD.

Results and discussion

Production and evaluation of Ri T-DNA-transformed sugar beet hairy roots in seedlings

With the purpose of evaluating the potential of RNA silencing to control BNYVV in genetically engineered sugar beet hairy roots, a highly conserved region of the viral replicase gene was selected as silencing target for the development of transgenic rhizomania resistance. Three RNA1 segments differing in size were inserted in an inverted repeat array, an arrangement capable of functioning as a strong silencing inducer (Johansen and Carrington 2001), into the binary vector pK7WIWG2 (II) (Karimi et al. 2002) using the Gateway technology. The resulting binary plant expression vectors GW-IR1, GW-IR2 and GW-IR3 were used for the generation of sugar beet seedlings with transformed hairy roots and non-transformed shoots and leaves.

Transformation was performed by inoculating *A. rhizogenes* strain R1000 at the hypocotyl of aseptically grown seedlings, resulting in the formation of adventitious kanamycin resistant roots approximately 8–10 days post transformation (Fig. 1b). A limited number of adventitious lateral roots appearing on the radicle section were occasionally observed in several seedlings, as a response to the removal of the root apical meristem (Fig. 1c). The emergence of such roots however, was recorded approximately 3–4 days following sectioning and was independent of *A. rhizogenes* transformation, since they were not produced from cells subjected to proper antibiotic pressure.

Hairy roots differed from wild type roots with respect to growth rate, structure and branching. Transgenic roots were profoundly abundant, accompanied by a large number of root extensions, a rapid growth rate and in some cases a plagiotropic development. Untransformed seedlings grown under no selection, developed roots of wild type phenotype approximately 15 days after removal of the existing root system. In contrast, those grown under antibiotic selection did not produce new roots and eventually showed necrosis at the wounded site of the hypocotyls (Fig. 1d). The frequency of cut hypocotyls producing kanamycin resistant hairy roots was 96% with an average number of 6.5 (range: 5–8) transformed roots per inoculated seedling.

To confirm the presence of the full-length transgene, kanamycin resistant roots were analyzed by a multiplex PCR targeting the BNYVV-derived transgene, the *npt*II gene and *virCD* of *A. rhizogenes*. Amplicons of the expected size were obtained for the BNYVV transgenes of 459, 589 or 824 bp, and *npt*II of 385 bp in all of the 60 roots examined (Fig. 2). The *virCD* fragment of 1,074 bp could not be amplified from roots, thus excluding the presence of *A. rhizogenes* and verifying the transgenic nature of hairy roots grown under kanamycin selection.
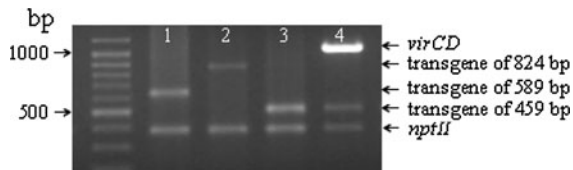
Fig. 2 Amplification products obtained by multiplex PCR for the evaluation of transgenic roots. *Lanes 1, 2, 3*: *A. rhizogenes*-transformed roots, carrying a BNYVV transgene (589, 824 and 459 bp, respectively) and the *nptII* gene (385 bp). *Lane 4*: *A. rhizogenes* cells transformed with GW-IR3, carrying the BNYVV-derived transgene of 459 bp. The amplicon of 1,074 bp corresponding to *virCD* of *A. rhizogenes* could only be amplified using bacterial cells as a template, thus verifying its absence in transgenic roots grown under kanamycin pressure. Ladder used: Gene Ruler Ladder mix (Fermentas)

Fig. 3 Symptoms of *Beet necrotic yellow vein virus* in sugar beet seedlings at 21 days post mechanical root inoculation. *I* Seedling with a GW-IR3 transgenic root system. *II* Seedling with vector-transformed root system. *III* Untransformed seedling with a wild type root system. Transgenic roots carrying a BNYVV replicase-derived hairpin molecule are symptomless, whereas vector-transformed and non-transformed seedlings, exhibit symptoms of root deterioration as well as leaf chlorosis and necrosis

Furthermore, BNYVV transgene expression was verified by means of a RT-PCR assay, in more than 90% of the 60 root samples examined (data not shown). These findings, combined with the observed inability of the untransformed seedlings to form roots under antibiotic pressure clearly indicated that selection of transformants was readily achieved on the basis of kanamycin resistance.

BNYVV replicase-derived dsRNA results in resistant hairy roots

Challenge-inoculation experiments were performed as a means to assess the ability of intron-hairpin constructs in inducing RNA silencing-based rhizomania resistance to Ri T-DNA-transformed sugar beet roots. Resistance was assessed by visual symptom observation as well as by measurement of virus titers through ELISA at 14 and 21 dpi. All together, 120 transformed seedlings for every intron-hairpin construct were included in three challenge-inoculation experiments. In untransformed control seedlings, stunting, leaf curling and sporadic chlorosis appeared at 16–20 dpi and approximately 95% of these plants exhibited systemic virus disease symptoms at 23–26 dpi (Fig. 3). The same symptom development was observed in challenge-inoculated seedlings transformed with the empty vector only, employed as a means to assess the performance of hairy roots expressing dsRNA originating from an unrelated source.

ELISA readings (OD_{405}) were in good agreement with these visual assessments: control plants

(untransformed and vector-transformed presented together) were found positive at 14 dpi and 115 out of 120 seedlings were fully infected at 21 dpi. As expected for BNYVV, the root system of each control seedling scored higher than its leaves. The reading values for roots at 14 dpi ranged from 0.437 to 0.607 whereas values for leaves ranged from 0.281 to 0.343 with the negative controls presenting values from 0.098 to 0.101 for both roots and leaves. The respective figures at 21 dpi were 0.80–1.15 for roots and 0.60–0.80 for leaves. Taken all together, the mean content of virus titer in the roots was significantly higher than that of the leaves in both test dates (Table 2).

The majority (>90%) of *A. rhizogenes*-transformed seedlings manifested a delay in symptom development of at least 7–10 days compared to control plants (Fig. 3). More than 90% of the 360 seedlings tested at 14 dpi, proved negative for BNYVV infection in both roots and leaves. The virus could be detected only in the leaves of a few seedlings (<10%) at a very low, marginally considered as positive ($OD_{405} = 0.28$–0.35) level, accompanied by the absence of visual symptoms. At 21 dpi, all seedlings had lower values in the roots than it the leaves. At this time, transformed hairy roots from more than 80% of the plants analyzed were found

Table 2 Mean of BNYVV ELISA values of three challenge-inoculation experiments

Constructs	ELISA readings (OD$_{405}$)				
	Number of seedlings tested	14 dpi		21 dpi	
		Roots	Leaves	Roots	Leaves
GW-IR1	120	0.127 (−)*	0.244 (−)	0.162 (−)	0.607 B**
GW-IR2	120	0.118 (−)	0.262 (−)	0.219 (−)	0.720 A
GW-IR3	120	0.111 (−)	0.201 (−)	0.116 (−)	0.483 C
Control***	120	0.522 a	0.312 b	0.998 a	0.709 b A

Data represent the average OD$_{405}$ readings of each seedling at 14 and 21 days post inoculation (dpi)

* (−) Value indicative of virus absence (reading for negative control: 0.098–0.102)

** Different letters denote statistically significant differences at $P = 0.05$. Small letters refer to horizontal comparisons between roots and leaves (paired t-test). Capital letters refer to comparisons among the four categories of means (three constructs and control) (F-test)

*** Average of untransformed and vector-transformed readings

completely virus-free while the rest presented marginally positive values (OD$_{405}$ = 0.26–0.33). At the same time the aerial, non-transformed parts of the same seedlings were found infected (OD$_{405}$ = 0.32–0.85). In addition, given that statistical analysis was meaningful in this case, significant differences among seedlings expressing the different transgenes were found, with those carrying the transgene of 459 bp presenting a better performance than the other two.

Collectively, these results indicate that a good, operational level of BNYVV-resistance was obtained in the transformed roots and further substantiate the conclusion that such resistance can be readily screened in genetically engineered hairy roots. The fact that hairy roots expressing the transgene of 459 bp performed better, as evidenced by the leaf values at 21 dpi, may relate to transgene stability; previous studies have also reported on the effect of the size of hairpin constructs in silencing induction capacity and further demonstrated that small hairpins are stronger silencing inducers than larger molecules (Heilersig et al. 2006).

As opposed to previously employed genes encoding for the coat protein or triple gene block proteins (Bleykasten-Grosshans et al. 1997; Andika et al. 2005), the conserved nature of the gene encoding the BNYVV replicase protein renders it a more suitable target for the purposes of achieving durable resistance against rhizomania disease of sugar beets. This kind of resistance, if expressed upon stable transformation and regeneration at the whole plant level, is expected to decrease virus spread in a transgenic crop under field conditions.

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