Polycistronic artificial miRNA-mediated resistance to *Wheat dwarf virus* in barley is highly efficient at low temperature

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SUMMARY

Infection of *Wheat dwarf virus* (WDV) strains on barley results in dwarf disease, imposing severe economic losses on crop production. As the natural resistance resources against this virus are limited, it is imperative to elaborate a biotechnological approach that will provide effective and safe immunity to a wide range of WDV strains. Because vector insect-mediated WDV infection occurs during cool periods in nature, it is important to identify a technology which is effective at lower temperature. In this study, we designed artificial microRNAs (amiRNAs) using a barley miRNA precursor backbone, which target different conservative sequence elements of the WDV strains. Potential amiRNA sequences were selected to minimize the off-target effects and were tested in a transient sensor system in order to select the most effective constructs at low temperature. On the basis of the data obtained, a polycistronic amiRNA precursor construct (VirusBuster171) was built expressing three amiRNAs simultaneously. The construct was transformed into barley under the control of a constitutive promoter. The transgenic lines were kept at 12–15 °C to mimic autumn and spring conditions in which major WDV infection and accumulation take place. We were able to establish a stable barley transgenic line displaying resistance to insect-mediated WDV infection. Our study demonstrates that amiRNA technology can be an efficient tool for the introduction of highly efficient resistance in barley against a DNA virus belonging to the Geminiviridae family, and this resistance is effective at low temperature where the natural insect vector mediates the infection process.

Keywords: artificial microRNA, *Hordeum vulgare*, leafhopper, low temperature, resistance, *Wheat dwarf virus*.

INTRODUCTION

Increasing biotic stresses caused by climate change are major challenges for worldwide crop production. Warmer autumns and

spring could have a positive effect on the spread and impact of plant diseases, presenting severe problems in crop production (Chakraborty *et al.*, 2000; Trebicki *et al.*, 2015). This is especially true for insect-transmitted plant viruses, where warmer temperatures could increase the migration potential of vector insects (Canto *et al.*, 2009). *Wheat dwarf virus* (WDV) is an economically important, leafhopper-transmitted virus belonging to the *Mastrevirus* genus (Geminiviridae family). WDV has a monopartite, single-stranded, circular DNA genome translating four viral proteins. The virion-sense V1 and V2 open reading frames (ORFs) encode the viral movement protein (MP) and coat protein (CP), whereas the other two ORFs, C1 and C2, located on complementary-sense, encode Rep and RepA proteins, respectively, associated with virus replication (Boulton, 2002). WDV is a pathogen of both wheat and barley, associated with chlorosis, severe stunting and even plant decline, causing severe yield losses in many countries (Ramsell *et al.*, 2009). There are two main types of WDV strain, barley and wheat strains, sharing approximately 84% nucleotide identity, which are able to infect the other host with low efficiency (Koklu *et al.*, 2007). As WDV is transmitted by leafhoppers (*Psammotettix alienus* Dhalbom; Hemiptera, Cicadellidea) in a circulative, non-propagative manner (Lindsten and Vacke, 1991), it is expected that the incidence of vector-mediated infection and virus spread will increase because of the longer and warmer autumn periods associated with climate change. *Psammotettix alienus* winters in egg stage; eggs hatch during April and the first adults appear in May or June depending on the temperature; *P. alienus* has two or three generations during the year in the temperate zone (Manurung *et al.*, 2005). During spring, the population of the first generation of leafhoppers starts to increase when the temperature reaches 15 °C, imposing a severe threat to barley plants (Lindblad and Sigvald, 2004). The most critical period is the late autumn when the abundance of leafhoppers is high and plants are in the early stages of development, rendering them susceptible to virus infection. The level of WDV infection has been shown to be positively correlated with the abundance of leafhoppers (Praslicka, 1997). The only measure to reduce yield losses is to combat the vectors using insecticides but, because of the abundance and mobility of leafhoppers, the success

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of this approach is limited. The production of tolerant/resistant cultivars would be the most environmentally sound and effective method to control the virus; however, only a few tolerant wheat cultivars have been identified so far, hampering the success of traditional breeding methods (Benkovics *et al.*, 2010).

To overcome the failure thus far of traditional breeding to deliver any resistance, biotechnological methods can be applied utilizing the efficient antiviral activity of RNA interference (RNAi) (Cillo and Palukaitis, 2014). RNAi is a conserved regulatory mechanism serving as an antiviral defence system (Seo *et al.*, 2013) and controlling endogenous gene expression (Chen, 2012). Virus infection triggers the production of virus-specific small interfering RNAs (siRNAs; 21 nucleotides in length) by the activity of RNAi machinery, which are then incorporated into the RNA-induced silencing complex (RISC), resulting in the sequence-specific degradation of the complementary viral RNAs (Pantaleo *et al.*, 2007). The expression of transgene-encoded long RNA fragments of viral sequences can be utilized to produce efficient resistance against viruses (Simon-Mateo and Garcia, 2011; Wang *et al.*, 2012). However, there are potential drawbacks associated with this technology. Processing of the double-stranded RNA (dsRNA) intermediate products of the expressed RNA by the RNAi machinery generates a population of siRNAs which can result in the manifestation of off-target effects, targeting unintended endogenous RNAs (Xu *et al.*, 2006). Another concern associated with the biotechnological utilization of the siRNA-mediated RNAi pathway is the potential inhibition of its activity at low temperature, as described in several species (Griesbach *et al.*, 2007; Hu *et al.*, 2011; Kasai *et al.*, 2009; Szittyia *et al.*, 2003). This temperature sensitivity might be caused by the temperature-dependent activity of plant RNA-dependent RNA polymerases involved in RNAi (Qu *et al.*, 2005), and can be bypassed by the transgenic expression of long dsRNA products (Di Nicola *et al.*, 2014; Hu *et al.*, 2011). microRNAs (miRNAs), similar to siRNAs, are small (21–22 nucleotides in length), non-coding RNAs that modulate gene expression by targeting mRNAs for degradation or translational repression (Bologna and Voinnet, 2014). However, in contrast with siRNAs, miRNAs are produced by sequential processing of genome-coded, long, single-stranded RNA molecules exhibiting highly specific stem-loop structures (Chen, 2009). The stem-loop structure (pre-miRNA) is recognized and cleaved by DICER-LIKE1 (DCL1), producing solely one short RNA duplex, from which one of the strands (mature miRNA) is incorporated into RISC, whereas the complementary strand (miRNA*) is usually degraded. An alternative approach to introduce virus resistance in plants is the application of artificial miRNA (amiRNA) targeting virus-specific sequences (Tiwari *et al.*, 2014). amiRNAs are designed by modifying the miRNA/miRNA* duplexes of an endogenous miRNA precursor to produce an amiRNA targeting experimentally defined target sequences (Parizotto *et al.*, 2004; Schwab *et al.*, 2006). The advantage of this technology is that the precise miRNA processing

facilitates to assess and hence reduce the potential off-target effects of the expressed small RNA in the donor plant. Moreover, as the miRNA pathway is an indispensable regulatory system governing developmental processes, it is expected that low temperature will not affect its activity. A pioneering work has demonstrated that amiRNA technology can be utilized as an efficient antiviral technology rendering *Arabidopsis thaliana* plants resistant to *Turnip yellow mosaic virus* and *Turnip mosaic virus* (Niu *et al.*, 2006), although viral escape was identified under the pressure of amiRNA-mediated silencing (Lin *et al.*, 2009; Simon-Mateo and Garcia, 2006). To reduce the risk of virus evolution resulting in the evasion of resistance, it is advantageous to use multiple amiRNAs targeting different conservative regions on the viral genome (Fahim *et al.*, 2012; Kung *et al.*, 2012; Zhang *et al.*, 2011). amiRNA-based virus resistance/tolerance has been successfully elaborated in model and crop plants to control the infection of *Plum pox virus* (Simon-Mateo and Garcia, 2006), *Cucumber mosaic virus* (Duan *et al.*, 2008; Qu *et al.*, 2007; Zhang *et al.*, 2011), *Potato virus Y* and *Potato virus X* (Ai *et al.*, 2011), *Wheat streak mosaic virus* (Fahim *et al.*, 2012), *Watermelon silver mottle virus* (Kung *et al.*, 2012) and *Tomato leaf curl New Delhi virus* (Vu *et al.*, 2013).

In this work, we utilized the power of amiRNA technology to construct WDV-resistant barley plants displaying highly efficient resistance at low temperature. Taking into account the conservative sequence elements of several WDV strains, we designed amiRNAs which were validated in a transient system to select the most efficient constructs. Barley plants were transformed with a polycistronic amiRNA construct expressing the best-performing amiRNAs. Infection studies using WDV carrying *Psammotettix alienus* vectors were carried out and the infected plants were kept at 12–15 °C to test the durability of resistance at low temperature. We identified transgenic barley plants showing extreme resistance against WDV in these experiments, and the level of resistance correlated with the expression level of amiRNAs. Our experiments revealed that amiRNA technology can control the replication of a DNA virus in barley, even at low temperature similar to field infection conditions. This biotechnological approach may have the potential to provide resistance sources for future barley breeding in the case of limited natural resistant resources.

RESULTS AND DISCUSSION

Construction of amiRNA backbone based on barley *hvu-MIR171* precursor

To construct an efficient barley-specific amiRNA backbone, we used the barley *hvu-MIR171* precursor [accession number: National Center for Biotechnology Information (NCBI), JX195502; mirBase, MI0016461]. This precursor possesses a compact, highly stable secondary structure which can facilitate the successful gen-

Fig. 1 Construction of modified barley microRNA (miRNA) precursor backbone suitable for artificial miRNA (amiRNA) production. (a) Secondary structures of the wild-type of the *hvu-MIR171* precursor (1) and the modified *hvu-MIR171MOD* precursor (2) with the two introduced cleavage sites (black arrows). The bold letters in the *hvu-MIR171MOD* precursor sequence indicate the three generated point mutations. The red letters show the mature *hvu-miR171* sequence. (b) Total RNA samples were prepared from *Nicotiana benthamiana* leaves agroinfiltrated with the *hvu-MIR171* (1) and *hvu-MIR171MOD* (2) precursors and the empty vector (M), and used for small RNA Northern blot hybridization assay employing the miR171-specific LNA probe. Relative gel loadings are indicated by ethidium bromide staining of ribosomal RNAs (rRNAs). (c) Schematic diagram of the one-step overlapping polymerase chain reaction (PCR) technique to construct the amiRNA vectors. The original miR171*/miR171 duplex (green and red) was replaced with the designed amiRNA*/amiRNA sequences (yellow and blue) in one-step PCR using primers F and R containing the introduced cleavage sites. The PCR product was cloned directly into the binary expression vector (pC61K) between the *Cauliflower mosaic virus* (CaMV) 35S promoter and terminator sequences.

eration of amiRNAs (Fig. 1a). To establish a fast and easy way to use the amiRNA tool, we introduced two restriction endonuclease recognition sites close to the mature and star strands, enabling the easy subcloning of the one-step polymerase chain reaction (PCR) mutagenesis product (Fig. 1a). Next, we tested the biological activity of the precursors using an *Agrobacterium*-mediated transient expression system. The wild-type (*hvu-MIR171*) and mutagenized (*hvu-MIR171MOD*) precursors were inserted into a binary vector under the control of the *Cauliflower mosaic virus* (CaMV) 35S constitutive promoter. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* harbouring the binary precursor constructs. Leaf samples were collected at 3 days post-infiltration and used for RNA analyses by small RNA Northern blotting. In both cases, we observed the accumulation of miR171 (Fig. 1b), indicating that the expressed precursors can be processed correctly by the RNA silencing machinery. The biologically active *hvu-MIR171MOD* precursor provides an easy way to use the amiRNA design tool requiring only a one-step PCR mutagenesis and subsequent ligation into the precursor expressing cassette (Fig. 1c).

Selection of WDV target sequences

The selection of appropriate amiRNA target sites in the virus genome is a prerequisite for the efficient activity of the generated

amiRNAs. Several criteria must be taken into consideration when selecting the target sites on the virus genome: (i) the sequence must fulfil the sequence composition and energetic requirements of the particular miRNA/miRNA* structure; (ii) biologically indispensable coding regions should be targeted; (iii) no potential off-target effects should be associated with activities of the amiRNAs; (iv) several target sites must be employed in parallel to avoid resistance breaking; and (v) the generated amiRNAs should be effective against a wide range of viral strains.

First, we applied the published amiRNA selection criterion to the WDV genome sequence to identify potential target sites (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>). The genome was screened with a 21-nucleotide-long window [C(or)GNNNNNNNNNNNNNNNNNNNA] in order to search for target sites fulfilling the basic energetic and sequence requirements of the potential amiRNA/amiRNA* duplexes; 136 and 31 potential target sites were identified in the Rep and MP coding sequences, respectively. To reduce the unintended effects, the potential amiRNAs targeting the identified target sites were investigated for potential off-target activities. Using an Institute for Genomic Research (TIGR) barley database (<http://plantgrn.noble.org/psRNATarget/>), we screened the available barley mRNAs allowing two or three mismatches for potential off-target effects. We found that 18 Rep and eight MP targeting candidate amiRNAs showed no off-target effect on barley

Overlapping PCR techniques were applied to generate amiRNAs by replacing the original miRNA/miRNA* duplex in the *hvu-MIR171MOD* construct (Fig. 1c). Ten amiRNAs based on the *hvu-MIR171MOD* backbone were designed to target the selected sequence elements. The natural miRNA/miRNA* sequences of *hvu-MIR171MOD* backbones were replaced with 10 customized amiRNA/amiRNA* sequences to generate 10 amiRNA precursors. The secondary structure and energetic parameters of the resident miRNA/miRNA* sequence were retained as much as possible in the modified precursors (Fig. 2). The RNA secondary structure of the amiRNA precursor constructs (designated amiR1–amiR10) was

Selection of the most efficient amiRNA constructs

The integration of helpful web-based miRNA designer tools and manual design facilitates the creation of target sequence-specific amiRNA candidates. However, in spite of the careful design, the *in vivo* efficiency of amiRNA candidates can be highly variable (Deveson *et al.*, 2013; Li *et al.*, 2013, 2014). This phenomenon is mediated by largely unpredictable and unknown factors associated with the expression, processing and activity of amiRNAs. Therefore, it is very important to apply a rapid *in vivo* screening method to evaluate the performance of the individual amiRNA candidates in order to enhance the success rate of the experimental techniques. To test the biological activity of our amiRNAs, the individual constructs were cloned into the pC61K binary vector under the control of the *CaMV* 35S promoter and transformed into *Agrobacterium tumefaciens*. Transient expression studies were carried out by infiltrating *N. benthamiana* leaves with *Agrobacterium* suspensions harbouring the individual amiRNA constructs. The infiltrated leaves were harvested at 3 days post-infiltration and processed for RNA extraction. Small RNA Northern blot analyses of RNA samples revealed that amiRNAs were expressed at very different levels in the transient assay. The expression levels of amiR3, amiR8, amiR9 and amiR10 were high compared with the moderate accumulation of amiR1, amiR2 and amiR6 (Fig. 3a). We were unable to detect the expression of amiR4, amiR5 and amiR7 in the transient assay. However, as the sensitivity of DNA oligonucleotide-based probes is low, we cannot exclude the low-level expression of these amiRNA constructs. These experiments showed that, although with different efficiency, seven of 10 amiRNAs were processed to the correct size at the detection level of the experiment. To reveal the biological activity of amiRNA candidates, we generated a transient *in vivo* sensor system by introducing the target sites of amiRNA into the 3' end of the green fluorescent protein (GFP) reporter gene. These sensor constructs were cloned into binary vectors under the control of the 35S promoter, rendering the sensors suitable for agroinfiltration studies (Fig. 3b). The sensor constructs designated pGFPamiR1-5 and pGFPamiR6-10 contain the 21-nucleotide-long perfect target sites in sequence for amiR1–amiR5 and amiR6–amiR10, respectively. *Agrobacterium*-mediated co-transformation experiments were carried out by co-infiltrating the GFP sensor constructs with the particular amiRNAs; hence, the inhibited activity of GFP reflects the activity of amiRNAs (Fig. 3c). In addition to the normal temperature (23 °C), we repeated the experiments at 15 °C to confirm the actions of amiRNAs at low temperature. GFP sensor constructs revealed that amiR1, amiR6, amiR7 and amiR8 act

Fig. 2 Predicted secondary structures of the artificial microRNA (amiRNA) precursors. The top panel indicates the predicted secondary structure of the *hvu-MIR171MOD* backbone. Eight (amiR1–8) and two (amiR9–10) amiRNAs were designed to target viral RNAs encoding Rep and/or RepA proteins (C1 and/or C2 genes) and movement protein (MP) (V1 gene), respectively. The amiRNA*/amiRNA duplexes are marked by red boxes and the red letters indicate the mature amiRNA sequences.

Fig. 3 Expression and biological activity of artificial microRNAs (amiRNAs) in transient assays. (a) Total RNA samples prepared from agroinfiltrated *Nicotiana benthamiana* leaves expressing amiRNA constructs (amiR1–10) and empty vector (M) were employed for small RNA Northern blot hybridization assay using specific DNA oligonucleotide probes. The Decade™ Marker RNA (Ambion) was used as molecular size marker (D). Relative gel loadings are indicated by the presence of endogenous miR159 detected by LNA oligonucleotide probe. (b) Schematic representation of the two pGFPamiR sensor constructs, each containing five [(1–5) and (6–10)] amiRNA target sites in the 3' untranslated region (UTR) of the *mGFP5* reporter gene, under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. (c) Biological activity of amiRNAs in transient co-infiltration assays at different temperatures. The pGFPamiR sensor constructs were co-infiltrated together with the corresponding amiRNA constructs into *N. benthamiana* leaves. As a control, the original pC61Khu-MIR171MOD (miR171) and either of the pGFPamiR sensor constructs were co-infiltrated. The reduced green fluorescent protein (GFP) fluorescence indicates biological activity of the individual amiRNAs, whereas the bright fluorescence indicates the disabled function of the amiRNAs. Three biological repeats of the transient assays displayed similar results.

efficiently at both temperatures. amiR5 at 23 °C and amiR9 at 15 °C show higher silencing efficiency. No or moderate silencing activity was associated with the expression of amiR2, amiR3, amiR4 and amiR10 (Fig. 3c). These experiments provided important data on the potential biological activity of amiRNA candidates, revealing that their ability to silence the GFP sensor does not correlate strictly with the expression level of amiRNAs (for example, amiR7), and that well-processed amiRNAs do not necessarily silence the sensor construct (amiR3 and amiR10). The GFP sensor constructs contain solely the target sequences; therefore, their flanking sequence is altered compared with the natural sequence, and we cannot exclude the possibility that target accessibility problems may modify the outcome of transient co-expression experiments (Gu *et al.*, 2012). It is important to note here that this technological approach is only valid when comparing the biological activity of different amiRNAs. However, the identification of amiRNA candidates showing high biological efficiency in these transient assays strongly increases their potentially successful function in stable transgenic plants. On the basis of the collected data, we selected the best-performing amiR1, amiR6 and amiR8, all targeting the replicase coding viral RNA, for the generation of the polycistronic amiRNA construct. The selection of

amiRNAs targeting the same RNA molecule at three different positions can significantly reduce the recombination-mediated breakdown of resistance. Moreover, it may further enhance the efficiency of the introduced resistance, as the targeted virus replicase is not only essential for virus replication but also acts as a viral suppressor of RNA silencing (VSR), disabling the host defence system (Wang *et al.*, 2014).

Generation of polycistronic amiRNA construct

Using the sequence information of the three selected amiRNAs (amiR1, amiR6 and amiR8), a synthetic construct (VirusBuster171) containing the amiRNA backbones in tandem repeats (Figs 4a and S3, see Supporting Information) was built by gene synthesis. The secondary RNA structure of the resulting polycistronic amiRNA precursor was predicted using the MFOLD program, which demonstrated that the individual amiRNA backbones fold correctly (Fig. S4, see Supporting Information). The polycistronic construct was cloned into the pC61K binary vector under the control of the CaMV 35S promoter and transformed into *Agrobacterium*. Using this construct and the described GFP sensors, transient co-expression studies were carried out by infiltrating

Fig. 4 Biological activity of the VirusBuster171 construct in transient assays. (a) Schematic representation of the structure of the polycistronic artificial microRNA (amiRNA) construct (VirusBuster171) under the control of the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter in a binary vector. (b) pGFPamiR sensor and VirusBuster171 (VB171) constructs were co-infiltrated into *Nicotiana benthamiana* leaves at different temperatures. As a control, the pC61Khvu-MIR171MOD (miR171) construct was co-infiltrated together with the sensors. Three biological repeats of the transient assays displayed similar results. (c) To express the biologically active amiRNA target, the Rep coding sequence from the WDV-h07 strain was cloned into a binary vector under the control of the constitutive CaMV 35S promoter (pRep). (d) Investigation of the biological activity of VirusBuster171 on its natural RNA target sequence encoding Rep. pRep was co-infiltrated together with either VirusBuster171 or an empty binary expression vector (pBIN). The co-expression of pRep and pGFP results in bright green fluorescent protein (GFP) fluorescence, as the viral suppressor of RNA silencing (VSR) activity of Rep inhibits RNA interference-mediated degradation of GFP mRNA. The co-expression of VirusBuster171 with pRep and pGFP results in reduced GFP activity, similar to the control infiltration (co-infiltration of pC61Khvu-MIR171MOD, pBIN and pGFP). Three biological repeats of the transient assays displayed similar results.

N. benthamiana leaves with *Agrobacterium* suspensions. Both GFP sensor constructs, one target site on pGFPamiR1-5 and two target sites on pGFPamiR6-10, were efficiently silenced at normal and low temperature, indicating that the amiRNAs in the polycistronic construct retained their biological activity (Fig. 4b). Next, we tested the biological activity of VirusBuster171 on the real viral target RNA. The WDV Rep coding sequence was cloned into a binary vector under the control of the 35S promoter (pRep; Fig. 4c) and transformed into *Agrobacterium*. As Rep also acts as a VSR, the co-infiltration with GFP expressing binary construct (pGFP) results in enhanced and prolonged GFP fluorescence (Fig. 4d). To test the activity of VirusBuster171 on the viral RNA target, we co-infiltrated pGFP and pRep. We found that VirusBuster171 was effective on the viral RNA target, as the observed reduced GFP fluorescence, similar to control infiltration, indicates the absence of VSR activity of Rep (Fig. 4d). This experiment also indicates that VirusBuster171 acts via the miRNA pathway, because it is expected that the VSR activity of Rep would inhibit the siRNA pathway.

Transgenic barley lines and resistance phenotyping following WDV challenge

On the basis of these results, we decided to produce stable barley transgenic lines harbouring the polycistronic construct. A monocotyledon-specific transformation vector was built by introducing the VirusBuster171 construct under the control of the maize polyubiquitin promoter (Fig. 5a). Barley plants (Golden Promise) were used for *Agrobacterium*-mediated transformation, as described previously (Bartlett *et al.*, 2008), and 77 transformants were collected from 20 independent calli. The expression level of amiRNAs was investigated by small RNA Northern blotting in transgenic barley T0 lines. We selected four lines displaying high (VB8), moderate (VB9 and VB10) and low (VB20) amiRNA expression (Fig. 5b). These lines were indistinguishable from the wild-type barley plants showing no phenotypical alterations. Transgenic and control plantlets were used for virus infection studies. To mimic the real field infection process, WDV-infected barley plants were produced by *Agrobacterium*-mediated delivery of the agroinfectious WDV clone into barley (Ramsell *et al.*, 2009). Then, the generated WDV-infected plants were used to feed the collected and propagated leafhoppers. After acquiring the virus, the leafhoppers were moved onto the transgenic lines and wild-type control plants placed in isolation chambers. This infection method reproducibly produces a 100% infection rate. The plants were kept at low temperature in the plant growth chamber (15 °C during the day and 12 °C at night) to produce environmental conditions similar to the infection process in nature. The transgenic and control barley plants were monitored by PCR analysis and Northern blotting for viral RNAs. At 42 days post-infection (DPI), the control plants showed signs of dwarfing and the appearance of virus-specific PCR and Northern

Fig. 5 Assessment of virus tolerance of transgenic barley lines after *Wheat dwarf virus* (WDV) infection. (a) Schematic diagram of the pCubiVirusBuster171 construct. (b) Northern blot analysis of the expression of artificial microRNAs (amiRNAs) in four barley lines (VB8, VB9, VB10, VB20) and a non-transformed control plant (M). After hybridization with amiRNA-specific probes, the membranes were stripped and rehybridized for miR159 as loading control. (c) Investigation of the virus content of transgenic and control (WDV1, WDV2) plants (a, young leaf; b, old leaf) by Northern blot hybridization and Rep-specific polymerase chain reaction (PCR) assays at four different time points [42, 56, 77 and 112 days post-inoculation (DPI)]. (d) Phenotypes of transgenic and control plants after WDV challenge at the indicated time points.

blot signals (Fig. 5c, d). At this time point, the VB20 line displayed moderate stunting and was PCR positive, but viral accumulation was below the detection level of Northern blot analyses. The other transgenic lines showed no virus accumulation. At later time points, the control plants showed typical and severe viral disease

symptoms and a high level of virus accumulation. The VB20 line exhibited a weak tolerance and accumulated a high level of the virus. The VB9 and VB10 lines, expressing VirusBuster171 at a moderate level, showed significant tolerance against the virus but, at 112 DPI, viral disease symptoms started to develop and PCR

Fig. 6 Expression of antiviral artificial microRNAs (amiRNAs) in T1 progenies. Total RNA extracts of 4-week-old T1 progeny plants of the VB8 T0 parental line were used for small RNA Northern blot analyses to detect the expression of amiR1, amiR6 and amiR8. Total RNA extracts from 4-week-old wild-type plants and the VB8 line were used as negative and positive controls, respectively. Relative gel loadings are indicated by the presence of endogenous miR159 detected by the LNA oligonucleotide probe.

analyses revealed the presence of the virus in these lines too. However, in spite of the clear viral disease symptoms, the level of the virus was below the detection level in Northern blot analyses. The VB8 line, which expressed VirusBuster171 at high level, showed no viral symptoms, and accumulation of the virus was not detected by either Northern blot analyses or PCR in this line. These data indicate that the VB8 line is fully resistant against WDV, even at low temperature. The comparative analyses of the lines demonstrate that the degree of tolerance/resistance is related to the expression level of amiRNAs. To test the heritability of the high-level expression of virus-specific amiRNAs from the VirusBuster171 construct, we collected seeds from the VB8 (T0) lines and investigated T1 progeny plants. The analyses of the T1 generation revealed that all of the 13 investigated plants displayed the accumulation of virus-targeting amiRNAs (Fig. 6). The absence of a null segregant in 13 T1 plants indicates that the original T0 plant contained multiple insertions of the transgene. Moreover, some of the lines showed an even higher level of amiRNA expression for all three amiRNAs than the parental VB8 line (Fig. 6). These data demonstrate that the introduced trait is stably heritable, mediating the high-level expression of antiviral amiRNAs.

CONCLUSION

Currently, no technologies to cure virus-infected plants are available, and the spread of viruses can be limited only by application of costly chemical treatments eliminating the virus-transmitting vector organisms. The best solution would be the cultivation of virus-resistant cultivars, but natural resistance resources against a number of viruses either do not exist or are not efficient. In this study, we demonstrated that, using polycistronic amiRNA technology, a long-lasting resistance can be established in barley against WDV, one of the most destructive and economically important viruses. We have developed an easy one-step method to generate virus-specific amiRNAs based on natural barley miRNAs, and have demonstrated that amiRNA selection can be optimized using a

filtering method. By simulating the natural infection process mediated by leafhoppers, we demonstrated that amiRNA-mediated resistance is effective at low temperature, which is extremely important as the infection can take place at low temperature under field conditions. In theory, our system is suitable for the targeting of different viruses or their strains simultaneously, and could be used not only in barley but also in wheat. To our knowledge, this is the first report describing amiRNA-mediated resistance against a DNA virus belonging to the Geminiviridae family in monocotyledons.

EXPERIMENTAL PROCEDURES

Plant materials and leafhoppers

Nicotiana benthamiana plants grown in soil under normal growth conditions in a growth chamber [Versatile Environmental Test Chambers; Sanyo, Tokyo, Japan; 14-h light (50 $\mu\text{E}/\text{m}^2/\text{s}$) and 10-h dark cycle at 23 °C] were used for agroinfiltration experiments. *Hordeum vulgare* cv. 'Golden Promise' plants were grown under 15 °C daytime and 12 °C night-time temperatures with a 16-h light (50 $\mu\text{E}/\text{m}^2/\text{s}$) and 8-h dark period to obtain material for *Agrobacterium*-mediated transformation. Transgenic barley plants were kept under the same conditions.

A pooled population of *P. alienus*, originally collected from a barley field, was maintained for several generations on barley (*H. vulgare* cv. 'MV Jubilent') kept in growth chambers under a 14-h light (50 $\mu\text{E}/\text{m}^2/\text{s}$) and 10-h dark cycle at 23 °C and 30%–40% relative humidity. Leafhoppers were maintained on potted plants covered with a fine mesh (mesh size, 0.1 mm).

Plasmid constructs

For expression of miRNAs or amiRNAs, the binary plasmid pC61K was built by introducing the *CaMV* 35S cassette from pBIN61S (Silhavy *et al.*, 2002) into pCambia2300 (Cambia, Canberra, Australia) using *Hind*III and *Eco*RI sites.

To isolate the barley *hvu-MIR171* precursor (MirBase accession number, MI0016461; NCBI accession number, JX195502), a 138-base pair-long fragment was amplified from a Golden Promise cDNA library using forward (*hvu-MIR171F*) and reverse (*hvu-MIR171R*) primers containing

KpnI and *XbaI* restriction sites, respectively. The PCR product was digested using *KpnI* and *XbaI*, and cloned into the pC61K binary vector.

To facilitate amiRNA production, two additional endonuclease recognition sites (5'-*Bst*II and 3'-*Mlu*I) were introduced into the *hvu-MIR171* backbone by the overlapping PCR primers *hvu-MIR171MODF* and *hvu-MIR171MODR*. The modified *hvu-MIR171* backbone (*hvu-MIR171MOD*) was cloned into pC61K, resulting in pC61Khvu-miR171MOD.

The amiRNAs were generated by PCR using the pC61Khvu-miR171MOD clone as template and amiRNA-specific oligonucleotides carrying sequence modifications (listed in Table S1, see Supporting Information). The PCR products were then cleaved with *Bst*II and *Mlu*I, and ligated into the pC61Khvu-miR171MOD binary plasmid cleaved with the same restriction enzymes to replace the wild-type stem-loop structure with the modified versions.

To generate the two amiRNA GFP sensor constructs, a 105-base pair-long PCR product generated by amplifying two, partially homologous, 59-nucleotide-long phosphorylated primers (amiR1-5targetF and amiR1-5targetR; amiR6-10targetF and amiR6-10targetR), containing either the recognition sites of amiRNAs 1–5 or 6–10, were ligated into the 3' untranslated region (UTR) of the *mGFP5* reporter gene in the pBIN61GFP (or pGFP in short as mentioned throughout this article) vector digested with *XbaI* and blunt ended with T4 DNA polymerase, resulting in sensor constructs of pGFPamiR1-5 and pGFPamiR6-10.

The polycistronic amiRNA construct (VirusBuster171) with the three selected amiRNAs (amiR1, amiR6 and amiR8) was artificially synthesized (GeneArt®, Life Technologies) (Fig. S3). For the *Agrobacterium*-mediated transient assay, the synthesized VirusBuster171 construct was ligated into the pC61K binary construct using the flanking *Bam*HI and *Pst*I restriction sites, resulting in pC61KVirusBuster171. For barley transformation, the pCambia1300 (Cambia, Canberra, Australia) vector, containing the *hptII* marker gene, was used. To generate a monocotyledon-specific transformation vector, a *Hind*III–*Bam*HI cleavage fragment from the pAHC25 plasmid (Christensen and Quail, 1996) was used to introduce the maize polyubiquitin promoter (Ubi) and its first intron into pCambia1300 to generate plasmid pCubi. Plasmid pAHC25 was also employed to amplify the *A. tumefaciens* nopaline synthase (NOS) terminator using NOSF and NOSR primers, and cloned into pCubi by *KpnI*–*SacI* digestion, resulting in pCubiNOS. The synthesized VirusBuster171 was cloned into pCubiNOS with *Bam*HI–*KpnI* digestion, resulting in the pCubiVirusBuster171 transformation vector.

The WDV-h07 barley strain (NCBI accession number, FM210034) was used to generate the head-to-tail agroinfectious clone, pPZP201WDV1,5MER, suitable for *Agrobacterium*-mediated virus infection, as described previously (Ramsell *et al.*, 2009).

The WDV Rep protein expressing binary plasmid was produced using the pPZP201WDV1,5MER clone as template by PCR with the WDVrepF and WDVrepR primers. The PCR product was cloned into the 35S cassette of pBIN61S using *XbaI*–*SacI* cleavage sites.

The PCR primers are listed in Table S1.

***Agrobacterium tumefaciens* infiltration**

The *A. tumefaciens* strain C58C1 [optical density at 600 nm (OD_{600}) = 1.0], harbouring the amiRNA, GFP and sensor constructs, was infiltrated into the leaves of young *N. benthamiana* plants according to the method

described previously (Varallyay *et al.*, 2010). In co-infiltration studies, binary constructs expressing pRep were diluted at OD_{600} = 0.3 and mixed with amiRNAs and sensor constructs in a 1 : 1 : 1 ratio. The infiltrated leaves containing the individual constructs were used for optical observation and Northern blot analyses.

RNA isolation and Northern blotting

Total RNA was extracted from agroinfiltrated and virus-infected plants at different time points using a TRI® Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA). For Northern blot analyses of viral RNAs, 5 µg of total RNA were separated on formaldehyde–1.2% agarose gels and blotted to Nytran NX membrane (Schleicher & Schuell, Dassel, Germany), as described previously (Varallyay *et al.*, 2014). A 413-bp-long PCR product amplified by the WDVrepDetF and WDVrepDetR primers was radioactively labelled using random priming probes by a Decalabel DNA labelling kit (Thermo Fisher Scientific, Vilnius, Lithuania) for WDV RNA detection.

Ten micrograms of total RNA from agroinfiltrated leaves were Northern hybridized to detect small RNAs, as described previously (Varallyay *et al.*, 2014), at 35 °C using amiRNA-specific DNA oligonucleotides as probes.

For small RNA Northern blot analyses of transgenic plants, the total RNA samples (10 µg) were fractionated on denaturing 12% polyacrylamide gels containing 8 M urea, blotted and cross-linked, as described previously (Pall *et al.*, 2007). Membranes were probed with radioactively labelled DNA oligonucleotide probes complementary to the mature amiRNAs. Five picomoles of each oligonucleotide probe were end labelled with [γ^{32} P]ATP using T4 polynucleotide kinase and hybridized to the membrane in ULTRAhyb-Oligo solution (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. MiR159-specific LNA oligonucleotide was used as internal control probe.

The PCR primers and oligonucleotides used in this study are listed in Table S1.

Barley transformation

The pCubiVirusBuster171 transformation vector was introduced into the AGL-1 strain and used for the transformation of the 1.5–2-mm-diameter immature barley embryos (Golden Promise), as described previously (Bartlett *et al.*, 2008); 386 embryos were treated with the AGL-1 *Agrobacterium* strain harbouring the vector constructs and 77 transformed plants were collected from 20 independent calli. DNA extracted from T0 plants using a DNeasy Plant Mini Kit (Qiagen, Germany) were applied for PCR (Phire Hot Start II DNA Polymerase) analyses to detect the presence of the *hptII* gen (hyg) and maize polyubiquitin (Ubi) promoter (data not shown) using the hygF and hygR or UbiDetF and UbiDetR primer pairs. The PCR primers are listed in Table S1.

WDV infection by infectious clone and leafhoppers

To produce WDV-infected barley plants, the AGL-1 *Agrobacterium* strain containing the pPZP201WDV1,5MER infectious clone was used to agroinfect overnight imbibed Golden Promise seeds, as described previously (Ramsell *et al.*, 2009). WDV-infected barley plants were used to feed the virus-free adult *P. alienus* stop-population in a micro-isolator chamber. After a week-long feeding, the infected leafhoppers were moved to 3–4-week-old test plants using two micro-isolator chambers with 3 insects per

isolator chamber for 1 week. Then, the insects of each chamber were pooled and PCR tested for the presence of WDV (Fig. S5, see Supporting Information) using the Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and WDVrepDetF and WDVrepDetR primers (Table S1).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Alignment of the replicase gene (C1) of 13 different *Wheat dwarf virus* (WDV) isolates (seven barley and six wheat strain) and the selected artificial microRNA (amiRNA; amiR1–8) target sites.

Fig. S2 Alignment of the movement protein gene (V1) of 13 different *Wheat dwarf virus* (WDV) isolates (seven barley and six wheat strain) and the selected artificial microRNA (amiRNA; amiR9–10) target sites.

Fig. S3 Sequence of the VirusBuster171 polycistronic artificial microRNA (amiRNA) construct synthesized by GeneArt® (Life Technologies). The amiRNA* sequences are indicated by yellow letters and the mature amiRNA sequences are indicated by blue letters. The flanking restriction endonuclease recognition sites are indicated by bold letters.

Fig. S4 Secondary structure of the VirusBuster171 construct generated by the MFOLD web server application (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>). The three mature artificial microRNA (amiRNA) sequences are indicated by blue lines.

Fig. S5 Polymerase chain reaction (PCR) analysis of the *Psammotettix alienus* insect vectors using the replicase gene-specific oligos (WDVRepDetF; WDVrepDetR) for the presence of *Wheat dwarf virus* (WDV). VB8, VB9, VB10 and VB20 are transgenic barley lines, whereas W1 and W2 are the wild-type controls infected by leafhopper vectors in two micro-isolator chambers (1 and 2). (K) is a negative control, (+) is the WDV-infected plant tissue as positive control and (M) is the molecular size marker (GeneRuler 1-kb ladder).

Table S1 The polymerase chain reaction (PCR) primer sequences used.