Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation

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Temperature dramatically affects plant–virus interactions. Outbreaks of virus diseases are frequently associated with low temperature, while at high temperature viral symptoms are often attenuated (heat masking) and plants rapidly recover from virus diseases. However, the underlying mechanisms of these well-known observations are not yet understood. RNA silencing is a conserved defence system of eukaryotic cells, which operates against molecular parasites including viruses and transgenes. Here we show that at low temperature both virus and transgene triggered RNA silencing are inhibited. Therefore, in cold, plants become more susceptible to viruses, and RNA silencing-based phenotypes of transgenic plants are lost. Consistently, the levels of virus- and transgene-derived small (21–26 nucleotide) interfering (si) RNAs—the central molecules of RNA silencing-mediated defence pathways—are dramatically reduced at low temperature. In contrast, RNA silencing was activated and the amount of siRNAs gradually increased with rising temperature. However, temperature does not influence the accumulation of micro (mi) RNAs, which play a role in developmental regulation, suggesting that the two classes of small (si and mi) RNAs are generated by different nuclease complexes.
Keywords: heat masking/miRNA/RNA silencing/siRNA/ temperature dependence

Introduction

RNA silencing is a sequence-specific post-transcriptional, gene-inactivation mechanism that operates in diverse eukaryotes. Accumulation of short (21–26 nucleotide), complementary guide RNAs corresponding to the sequence of target RNA occurs during RNA silencing in plants and animals (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Parrish *et al.*, 2000). These complementary guide RNAs provide the sequence-specificity of target RNA silencing. The double-stranded (ds) RNA-specific enzyme, DICER, produces two types of guide RNAs, small interfering (si) RNAs and micro (mi) RNAs. siRNAs are ds molecules generated from long

dsRNAs and mediate defensive responses against molecular parasites [referred to as post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animals] (reviewed in Voinnet, 2001; Zamore, 2001; Hannon, 2002). miRNAs are single-stranded molecules excised by DICER from short hairpin precursors and miRNAs are hypothesized to play a critical role in both animal and plant developmental regulation (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Llave *et al.*, 2002; Reinhart *et al.*, 2002). Both siRNAs and miRNAs incorporate into a multicomponent nuclease complex (RISC) and guide RISC to target homologous mRNAs for cleavage at near perfect base-pairing (Elbashir *et al.*, 2001; Hutvagner and Zamore, 2002), or for translational repression at partial complementarity (Reinhart *et al.*, 2000). In plants, siRNAs might also act as the specificity determinants for other complexes, including dsRNA amplification by RNA-dependent RNA polymerase (RdRP), systemic silencing (see below) and RNA-directed methylation of homologous DNA (reviewed in Waterhouse *et al.*, 2001; Mlotshwa *et al.*, 2002).

A good deal of evidence suggests that RNA silencing plays a natural role in plant defense against molecular parasites, including viruses (reviewed in Voinnet, 2001). Virus-induced RNA silencing operates at both single-cell and whole-plant levels. Accumulation of dsRNA intermediates of virus replication in a plant cell induces silencing activity, which degrades the dsRNA itself and the cognate mRNAs (referred to as cell-autonomous silencing). Cell-autonomous silencing also generates a mobile signal that triggers the degradation of homologous mRNAs in distant cells (systemic silencing) (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997). Consistent with the antiviral nature of RNA silencing in plants, many plant viruses have evolved silencing-suppressor proteins that block cell-autonomous or systemic silencing (Li and Ding, 2001). Transgene expression can also trigger RNA silencing. Virus- and transgene-induced silencing pathways are similar except that transgene-derived dsRNAs are likely generated by a plant encoded RdRP from aberrant transcripts. Transgene-induced RNA silencing is widely used in plant biotechnology. Phenotypes of virus-resistant plants expressing virus-derived sequences and transgenic plants, in which an endogenous gene is inactivated by ectopic expression of sense or antisense RNAs, are based on transgene-induced RNA silencing (Di Serio *et al.*, 2001; Rovere *et al.*, 2002).

Plant–virus interactions are strongly modified by environmental factors; especially by temperature. High temperature is frequently associated with attenuated symptoms ('heat masking') and with low virus content of virus-infected plants (Johnson, 1922; Hull, 2002). In contrast, rapid spread of virus diseases and the development of severe symptoms are frequently associated with

cold air temperature (Hine *et al.*, 1970; Gerik *et al.*, 1990). Harrison (1956) has speculated that the virus content of a plant represents an equilibrium between replication and degradation and that the activity of the virus degrading system increases with temperature. However, the underlying molecular mechanisms of these effects of temperature are still missing. We hypothesized that RNA silencing might be the postulated virus degradation system and temperature modifies plant–virus interactions through the regulation of RNA silencing.

Here we report that RNA silencing-mediated plant defence is temperature dependent. At low temperature, both virus- and transgene-induced RNA silencing is inhibited leading to enhanced virus susceptibility and to loss of silencing-mediated transgenic phenotypes. Furthermore, at low temperature, the level of virus- or transgene-derived siRNAs is dramatically reduced. In contrast, RNA silencing is activated and the amount of siRNAs gradually increases with temperature rising. However, accumulation of miRNAs, which play a critical role in developmental regulation, is temperature independent, suggesting that the two classes of small (si and mi) RNAs are generated by different nuclease complexes in plants.

Results and discussion

Temperature regulates RNA silencing-based antiviral defence by the control of siRNA generation

To test whether RNA silencing might correspond to the suggested temperature-dependent virus degradation system (Harrison, 1956), we analysed the temperature dependence of virus-induced RNA silencing at both cellular and whole-plant levels. To define the role of temperature in virus-induced cell-autonomous silencing, accumulation of virus-specific siRNAs was analysed in *Nicotiana benthamiana* protoplasts transfected with *Cymbidium* ringspot virus (CymRSV) at different temperatures (15, 21, 24 and 27°C). Cym19stop mutant virus, which is unable to express CymRSV encoded p19 silencing suppressor (Szittya *et al.*, 2002), was also used for protoplasts transfection in order to avoid the effect of silencing suppression. Viral RNA and siRNA levels were comparable in Cym19stop- and CymRSV-infected protoplasts at each temperature (Figure 1) confirming that p19 does not affect virus-induced local silencing (Silhavy *et al.*, 2002). Striking differences were found, however, in virus-derived siRNA accumulation at different temperatures; siRNAs were abundant at 27°C, but undetectable at 15°C. The amount of siRNAs gradually increased with temperature from 21 to 27°C (Figure 1). These findings indicate that virus-induced cell-autonomous silencing is temperature dependent, and suggest that at low temperature the silencing pathway is inhibited at or before the production of siRNAs. Since virus-specific siRNAs mainly derive from the dsRNA intermediates of viral replication (Voinnet, 2001), and since CymRSV still replicates efficiently at 15°C (Figure 1), it is likely that DICER activity is compromised at low temperature.

Previously, we showed that the CymRSV-encoded p19 acts as a viral suppressor of systemic silencing, thus Cym19stop-infected plants recover from viral infection at
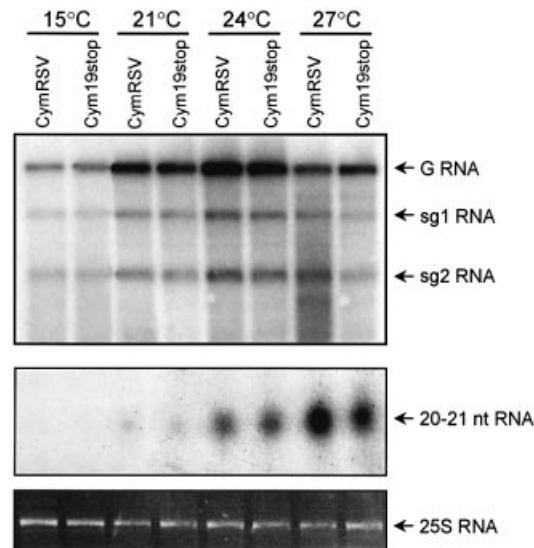

Fig. 1. Effect of temperature on virus-induced cell-autonomous RNA silencing. RNA gel blot analysis of RNA samples extracted from *N.benthamiana* protoplasts infected with *in vitro* transcript of CymRSV and Cym19stop. Transfected protoplasts were incubated for 24 h under a 14 h light and 10 h dark regime at different temperatures. G shows genomic RNA, while sg1 and sg2 refer to subgenomic 1 and subgenomic 2 RNAs, respectively. The size of siRNAs were estimated using labelled 21 nucleotide synthetic RNAs, and 25S RNA was applied as loading control.

standard temperature (21°C; Figure 2A) (Silhavy *et al.*, 2002). To test the effect of temperature on virus-induced systemic RNA silencing, *N.benthamiana* plants were inoculated with CymRSV and Cym19stop viruses and grown at different temperatures. As expected, CymRSV-infected plants died within 2 weeks at 15, 21 and 24°C (Figure 2A). Consistent with previous results (Gerik *et al.*, 1990), we found that CymRSV symptoms were 'heat masked' at 27°C (Figure 2A) and that the attenuated symptoms were associated with reduced virus level (Figure 2B). Plants infected with the Cym19stop showed the expected recovery phenotype at 21 and 24°C (Szittya *et al.*, 2002). Strikingly, at 27°C, the mutant virus was unable to infect the plants (Figure 2), while at 15°C, Cym19stop-infected plants displayed strong viral symptoms (Figure 2A). This latter result demonstrated that at low temperature, RNA silencing failed to protect the plants even when the virus lacked a silencing suppressor. It is believed that in many incompatible plant–virus interactions, plants are protected by RNA silencing-mediated defence (Voinnet, 2001). However, at low temperature, these interactions might become compatible and allow viruses to spread and accumulate in plants.

In situ hybridization analysis revealed (Z.Havelda, unpublished data) that at 24°C, CymRSV and Cym19stop viruses accumulated to similar levels in the infected cells of the first non-inoculated leaves that showed viral symptoms (systemically-infected leaves). However, the spread of CymRSV and Cym19stop were dramatically different. CymRSV invaded the whole systemically-infected leaf, while Cym19stop was confined close to the veins, presumably due to systemic silencing. To monitor systemic silencing at different temperatures, we compared the virus extent in the first systemically-
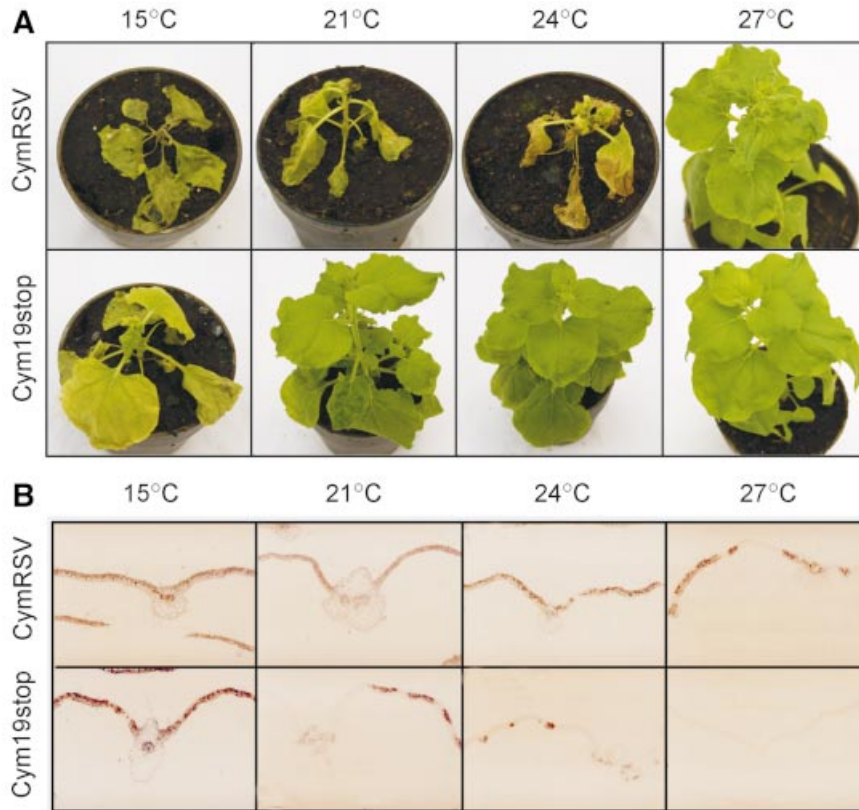
Fig. 2. Effect of temperature on symptom development and virus spreading of virus-infected plants. Virus-infected *N.benthamiana* plants were grown at the indicated temperatures. (**A**) Symptoms of CymRSV- and Cym19stop-infected plants at 14 dpi. (**B**) Spread of CymRSV and Cym19stop in the first systemically-infected leaves at 7 dpi. Digoxigenin-11-UTP-labelled RNA probe complementary to the last 900 nucleotides of CymRSV was hybridized to tissue sections and detected with alkaline phophatase-conjugated anti-digoxygenin antibody.

infected leaves of both CymRSV- and Cym19stop-inoculated plants. As Figure 2B shows, at 21°C as well as at 24°C, the spread of Cym19stop was blocked whereas CymRSV invaded the whole leaf. By contrast, at low temperature (15°C), both mutant and wild-type viruses invaded the entire first systemically-infected leaves. At 27°C, no virus accumulation was detected in systemic leaves of Cym19stop-inoculated plants. Moreover, RNA gel blot analyses failed to detect Cym19stop RNA in the inoculated leaves (data not shown), presumably because the mutant virus was restricted in the initially-infected cells. The spread of wild-type CymRSV was also limited at 27°C (Figure 2B), suggesting that a hyper-activated systemic silencing can protect plants even from highly invasive viruses.

Thus, virus-induced RNA silencing is a temperature-dependent RNA degradation mechanism that operates inefficiently at low temperature and whose activity increases with temperature. Since the lack of RNA silencing enhances plant susceptibility to viral infection (Mourrain *et al*., 2000; Dalmay *et al*., 2001), we suggest that the frequent outbreaks of plant virus diseases in unusually cold seasons (Hine *et al*., 1970; Gerik *et al*., 1990) reflect reduced RNA silencing capacity, while heat masking is due to an increased RNA silencing activity. Moreover, the efficiency of heat therapy (growing plants at 35–40°C to free from viruses) could also be explained as reflecting a hyper-activated RNA silencing activity.

Low temperature inhibits transgene-induced RNA silencing

In plants, transgene- and virus-induced RNA silencing pathways are overlapping but not identical (Voinnet, 2001). To test whether transgene-induced silencing is also temperature dependent, *Agrobacterium* infiltration (agroinfiltration) assays were carried out at different temperatures. Infiltration of non-transgenic *N.benthamiana* leaves with a strain of *Agrobacterium tumefaciens* carrying a GFP gene construct (35S-GFP) results in transient GFP expression but also leads to induction of GFP RNA silencing (Johansen and Carrington, 2001; Silhavy *et al*., 2002). Decline in GFP mRNA levels and accumulation of GFP-specific siRNAs in the infiltrated patches reflect the intensity of transgene-induced cell-autonomous RNA silencing. As Figure 3A shows, at 3 days post-infiltration (dpi), GFP mRNA levels were high at 15°C and decreased with the increase of temperature. To clarify whether the temperature-dependent GFP mRNA levels reflect the different silencing or different transcriptional activity, 35S-GFP was co-infiltrated with p19 (35S-p19), a potent inhibitor of transgene-induced RNA silencing (Silhavy *et al*., 2002), and the infiltrated plants were incubated at different temperatures. Since in the lack of RNA silencing GFP mRNA levels were equally high at any temperature (Figure 3A), we concluded that temperature-dependent GFP mRNA accumulation reflected the different silencing activities. Consistently,
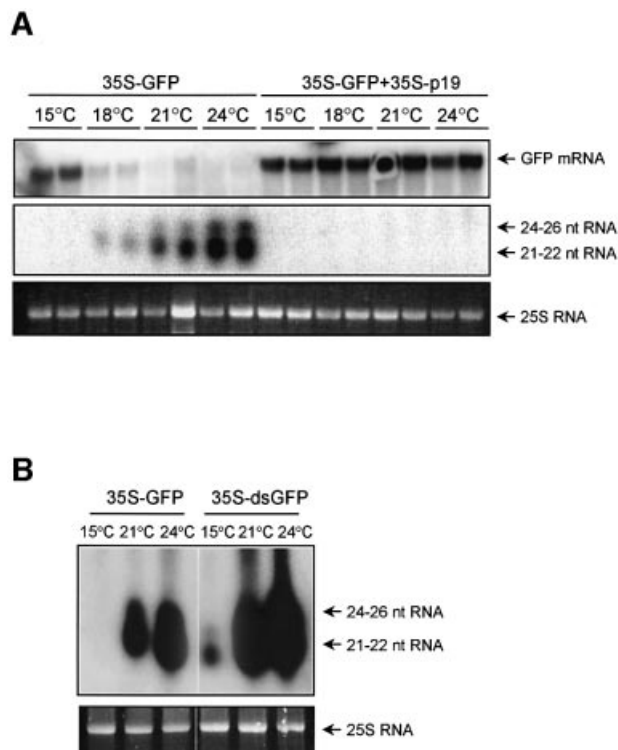
A

B

Fig. 3. Effect of temperature on transgene-induced RNA silencing. Non-transgenic *N.benthamiana* leaves were infiltrated with an *Agrobacterium* expressing 35S-GFP (**A** and **B**, left panels) or 35S-dsGFP (**B**, right panel), thereby inducing GFP silencing, or co-infiltrated with 35S-GFP and 35S-p19 silencing suppressor (A). Plants were further grown at different temperatures as indicated. RNA gel blot analysis was carried out to monitor the accumulation of GFP mRNA and GFP-specific siRNAs at 3 dpi. Total RNAs were isolated from infiltrated plants grown at the indicated temperatures under a 14 h light and 10 h dark regime. 25S ribosomal RNA was applied as loading control.

GFP-specific siRNAs did not accumulate at 15°C, but were easily detectable at 18°C, and siRNA levels increased gradually with temperature (Figure 3A). We also repeated the infiltration experiment using GFP-expressing *N.benthamiana* plants (Voinnet *et al.*, 1998). Similarly to the results obtained by agroinfiltration of wild-type, non-trangenic plants, GFP mRNAs accumulated to a high level, while siRNAs were not detectable in the infiltrated patches at 15°C. However, with the temperature rising, siRNA levels gradually increased displaying correlation with the GFP mRNAs degradation. (see Supplementary figure, available at *The EMBO Journal* Online). RNA silencing induced by agroinfiltration leads to accumulation of two distinct siRNA size classes, a 21–22 nucleotide short and a 24–26 nucleotide long fraction. We found that levels of short and long siRNAs increased simultaneously with temperature. Short siRNAs might mediate mRNA degradation, while long siRNAs are supposed to play a role in systemic silencing and methylation of homologous DNA (Hamilton *et al.*, 2002). To investigate whether processing of dsRNA to siRNAs in transgene-induced RNA silencing is temperature dependent, plants were infiltrated with an *Agrobacterium* carrying an inverted repeat of the GFP sequence thereby expressing dsGFP RNA (35S-dsGFP).

Since cleavage of dsRNAs into siRNAs does not require RdRP activity (Beclin *et al.*, 2002), low siRNA levels in 35S-dsGFP-infiltrated plants at 15°C (Figure 3B), suggest that cleavage of dsRNA to siRNAs is temperature dependent. However, we still do not know whether RdRP-mediated dsRNA generation is temperature dependent or not. An unlikely, alternative explanation for low siRNA accumulation is that siRNA stability is greatly reduced at low temperature (discussed later).

RNA silencing-based transgenic phenotypes are lost at low temperature

RNA silencing has enormous potential in plant biotechnology to confer protection against viruses and to inactivate endogenous genes. Since both virus- and transgene-induced RNA silencings are temperature dependent, we tested the stability of RNA silencing-mediated transgenic phenotypes at different temperatures.

To examine the effect of temperature on engineered virus resistance, transgenic *N.benthamiana* plants expressing a CymRSV-derived RNA (92KA1; Rubino and Russo, 1995) were inoculated with CymRSV and incubated at different temperatures. We confirmed that transgenic plants were resistant at 24°C and that the resistance was based on RNA silencing (Rubino and Russo, 1995; data not shown). However, at 15°C, strong symptoms developed and CymRSV RNA accumulated to a high level in the inoculated plants (Figure 4), demonstrating that the transgene-mediated virus resistance was broken at low temperature. Our results predict previously unanticipated failure in virus resistance at lower temperature for transgenic crops with RNA silencing-mediated virus resistance.

The effect of temperature on antisense-mediated endogen gene inactivation was examined in the model species *Arabidopsis* and in the economically important potato, in which antisense inhibition of genes involved in carbohydrate metabolism is widely applied. *Arabidopsis* plants (anti-AtHXK1) expressing antisense hexokinase (HXK1) RNAs are sugar hyposensitive, allowing anti-AtHXK1 plants to grow at limiting glucose (6%) concentration (Jang *et al.*, 1997). Seedlings of anti-AtHXK1 and wild-type plants were grown on agar plates containing 6% glucose at 15°C and 24°C. As expected, at 24°C development of wild-type plants was inhibited, while anti-AtHXK1 plants developed normally (Figure 5A). Accumulation of transgene-derived siRNAs in anti-AtHXK1 plants at 24°C indicated that HXK1 mRNAs were inactivated by RNA silencing (Figure 5A). At 15°C, the development of both wild-type and anti-AtHXK1 plants was inhibited, showing that the antisense inhibition was not effective at low temperature. Consistently, transgene-derived siRNAs could not be detected in anti-AtHXK1 plants at 15°C (Figure 5A).

Transgenic potato plants displaying antisense inhibition of StubGAL83 mRNAs (A.Lovas, unpublished data) were also analysed at different temperatures. At 24°C, high levels of StubGAL83-specific siRNA accumulation were associated with extremely low StubGAL83 mRNA levels. In contrast, at 15°C, StubGAL83 mRNA accumulated to a high level and transgene-derived siRNAs were barely detectable (Figure 5B). These observations demonstrate that StubGAL83 inactivation is mediated by RNA
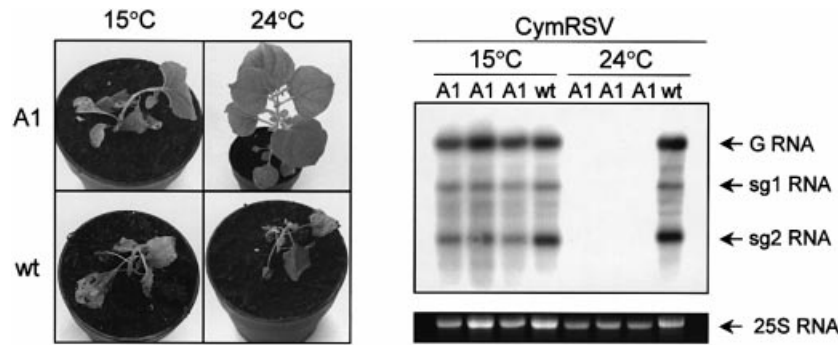
Fig. 4. Effect of temperature on RNA silencing-mediated transgenic virus resistance. CymRSV 92KA1 *N.benthamiana* transgenic plants (A1) expressing a CymRSV RNA segment, which confers CymRSV resistance, and control non-transgenic plants (wt) were infected with CymRSV transcript. Virus-infected plants were incubated at the indicated temperatures. Symptoms (left panel) and viral RNA levels (right panel) of infected plants at 10 dpi. G shows genomic RNA, while sg1 and sg2 refer to subgenomic 1 and subgenomic 2 RNAs, respectively. 25S ribosomal RNA was applied as loading control.
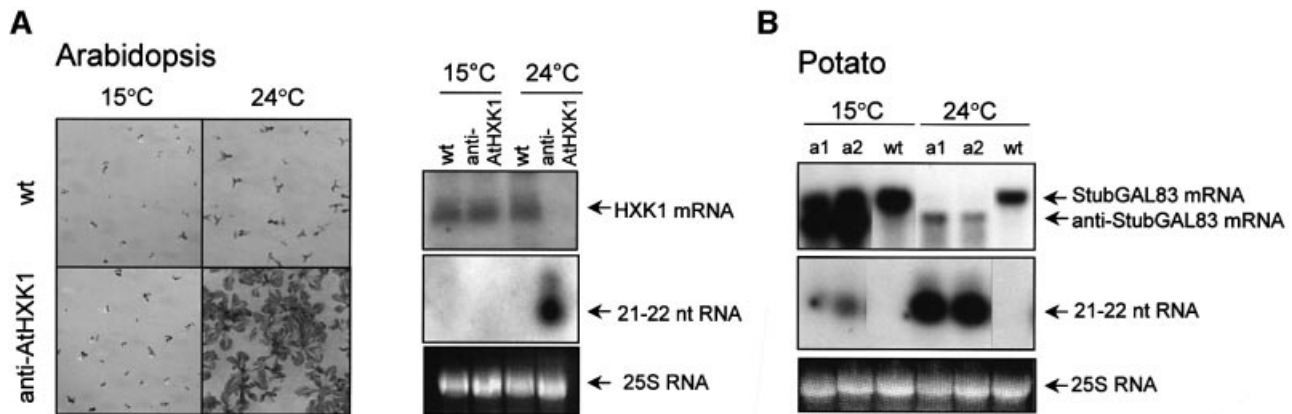

Fig. 5. Effect of temperature on antisense RNA-mediated inhibition of endogenous genes. Seedlings of transgenic *Arabidopsis* (anti-AtHXK1) expressing antisense RNA for hexokinase (HXK1) and wild-type plants were grown for 3 weeks on agar plates containing 6% glucose at 15°C and 24°C (**A**, left panel). Accumulation of mRNAs and specific siRNAs in transgenic anti-AtHXK1 expressing antisense RNA for hexokinase (HXK1) mRNA (A, right panel), and in two transgenic potato lines (aG1 and aG2) expressing antisense RNA (anti-StubGal83 RNA) for Gal83 mRNA (StubGal83 RNA) (**B**). Plants were incubated at 15°C and 24°C. Northern blots were hybridized with a strand-specific probe to HXK1 mRNA and ds StubGAL83 cDNA, respectively. 25S ribosomal RNA was applied as loading control.
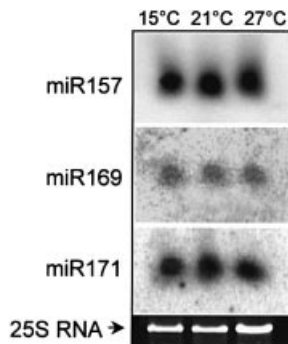

Fig. 6. miRNA accumulation in *Arabidopsis*. Accumulations of miRNAs were analysed in *Arabidopsis* seedling grown *in vitro* at different temperatures. RNA gel blots were hybridized at 37°C using the 5′-labelled anti-sense DNA oligos corresponding to the indicated miRNAs. 25S ribosomal RNA was applied as loading control.

silencing in antisense expressing plants, and that this RNA silencing is compromised at low temperature in potato.

Since RNA silencing-mediated transgenic phenotypes could be lost at low temperature, biotechnological application of these transgenic plants has a considerable environmental limit. This constrain could be especially important for perennials and less limiting for annual crops. Inefficient RNA silencing activity at low temperature might be useful to express foreign proteins to a high level in transgenic plants. Moreover, inefficient RNA silencing activity at low temperature could also be utilized in RNA silencing-based genetic screens, since silencing-mediated early lethal phenotypes could be rescued at low temperature. Indeed, in plants, RNA silencing-based male sterility was rescued by lower temperature (Mou *et al*., 2002).

Accumulation of miRNAs are not affected by temperature

In animals, DICER is required for both siRNA and miRNA production (Grishok *et al*., 2001; Hutvagner *et al*., 2001). The DICER homologue, CARPEL FACTORY is required for miRNA generation in plants (Reinhart *et al*., 2002). Since siRNA accumulation is temperature dependent in plants, we tested whether miRNA accumulation is also controlled by temperature. In contrast to siRNA production, miR157, miR169 and miR171 RNAs accumulated to equal levels at 15, 21 and 24°C in *Arabidopsis* (Figure 6), probably explaining why the plants developed normally at all three temperatures. These findings suggest that plant
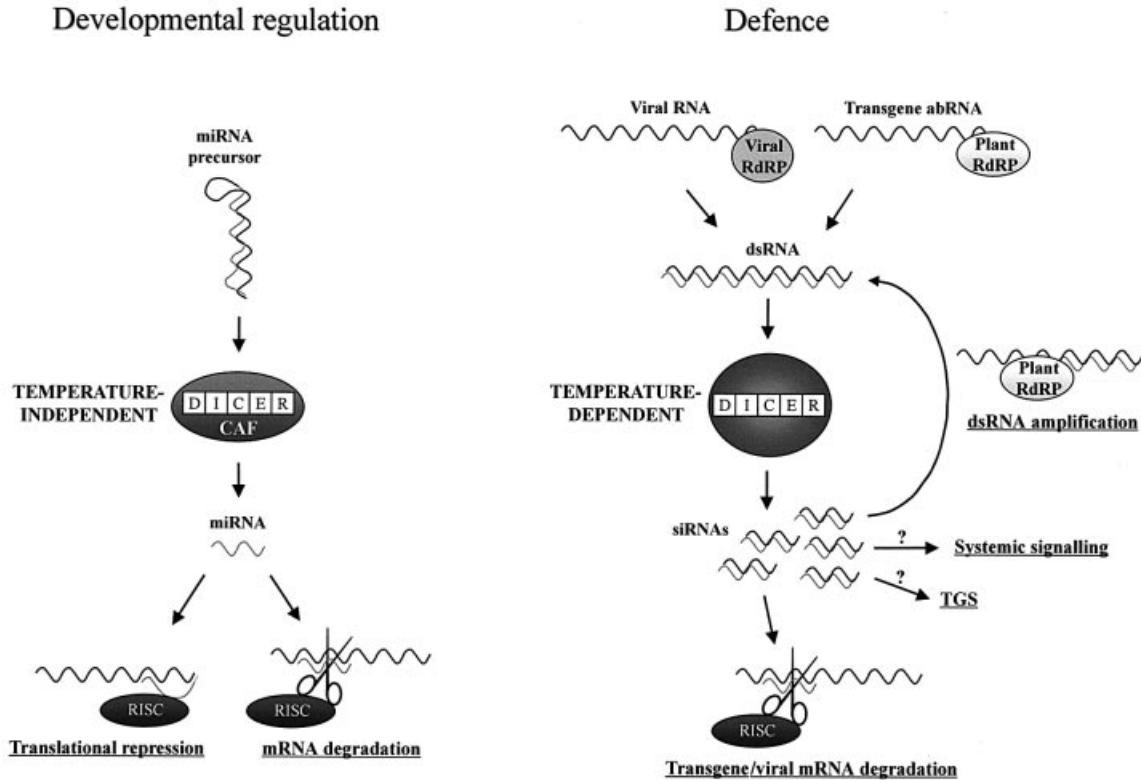
RNA silencing

Developmental regulation

Defence

Fig. 7. Model for RNA silencing pathways in plants. RNA silencing is branched into temperature-independent regulatory and temperature-dependent defensive pathways. siRNA generation is temperature dependent, thus RNA silencing-based defence responses are affected by temperature. Since miRNA production is temperature independent, developmental regulation is not affected by low temperature.

siRNAs and miRNAs are generated by different pathways, and probably by distinct DICER-like complexes. At least four DICER-related genes can be identified in the *Arabidopsis* genome (Reinhart *et al.*, 2002); it is conceivable that a temperature-dependent DICER homologue plays role in siRNA generation and a temperature-independent DICER-related gene is involved in miRNA production. Alternatively, any component of the DICER complex that is specifically required for siRNA generation might be temperature dependent (Tabara *et al.*, 2002). Both siRNAs and miRNAs incorporate into the same RISC complex (Elbashir *et al.*, 2001), therefore we do not favour the alternative explanation that siRNAs are degraded at low temperature while miRNAs are not. Since plant siRNAs and miRNAs might act in an identical silencing pathway by guiding RISC to cleave target genes (Rhoades *et al.*, 2002), the temperature-dependent antiviral role and the temperature-independent developmental regulatory function of RNA silencing machinery may be separated at their respective small RNA-generating steps (Figure 7).

Conclusions

We find that siRNA-mediated RNA silencing is temperature dependent in three dicot species, thus inefficient siRNA generation at low temperature is probably a universal feature of higher plants. We speculate that

separation of siRNA and miRNA production in plants is a part of a general adaptive response. We suggest a model (Figure 7) in which, at low temperature and perhaps under other unfavourable conditions, the non-essential RNA silencing machinery is inactivated, but miRNA-mediated essential regulatory functions continue to operate, ensuring normal plant growth. As a cost, plants are more susceptible to viruses and to other molecular parasites at low temperature. In animals and *Chlamydomonas*, RNA silencing-mediated defence pathways are involved in inactivation of mobile elements. In plants, siRNAs are supposed to provide sequence specificity for a complex that mediates transcriptional gene silencing (TGS) by directly methylating homologous DNA segments (Mette *et al.*, 2001; Hamilton *et al.*, 2002) or initiating DNA methylation by imposing histone H3 lysine-9 methylation (Gendrel *et al.*, 2002; Volpe *et al.*, 2002). At low temperature, siRNA-mediated methylation might decrease, leading to the re-expression of transcriptionally silenced genes. Consistently, the general level of DNA methylation is reduced in plants at low temperature and TGS is less efficient (Finnegan *et al.*, 1998). Furthermore, the *Antirrhinum* Tam3 transposon is excised at 15°C, but not at 25°C, and transposition correlates with reduced methylation status of mobile element (Kitamura *et al.*, 2001). We can also speculate that inefficient siRNA

generation at low temperature might be involved in certain developmental regulation steps, for instance, in vernalization cold results in demethylation of regulatory genes (Reeves and Coupland, 2000).

Materials and methods

Plant materials

Wild-type *N.benthamiana* plants grown in soil under normal growth conditions were used for protoplast preparation, virus infection and agroinfiltration. For temperature treatment, after infection or infiltration, plants were grown in fitotron (Versatile Enviromental Test Chambers; Sanyo, Tokyo Japan) under a 14 h light (50 µE m^{-2}s^{-1}) and 10 h dark regime at different temperatures. The 92KA1 transgenic *N.benthamiana* plants were grown as described previously (Rubino *et al.*, 1993). For temperature treatment, after infection with CymRSV, 92KA1 transgenic plants were grown in fitotrons under a 14 h light (50 µE m^{-2}s^{-1}) and 10 h dark regime at 15 and 24°C, respectively. For temperature treatment, the AtHXK1 transgenic *Arabidopsis* (Jang *et al.*, 1997) and the control (wild-type) lines were germinated and grown in fitotrons under constant white light (100 µE m^{-2}s^{-1}) for 3 weeks on half-strength Murashige and Skoog plates containing 6% glucose at 15 and 24°C, respectively. For temperature treatment, stem segments from the anti-StubGAL83 transgenic potato and the control (wild type) lines were grown in fitotrons under a 14 h light (50 µE m^{-2}s^{-1}) and 10 h dark regime for 2 months on RM medium at 15 and 24°C, respectively. To determine the miRNA level, *Arabidopsis* plants were germinated and grown in fitotrons under a 14 h light (100 µE m^{-2}s^{-1}) and 10 h dark regime for 3 weeks on half-strength Murashige and Skoog plates at 15 and 24°C, respectively.

Agrobacterium tumefaciens infiltration

The *A.tumefaciens* infiltration method was carried out according to Voinnet *et al.* (1998) except that both non-transgenic and GFP-expressing transgenic plants were used. For co-infiltration, an equal volume of indicated *A.tumefaciens* cultures (OD$_{600}$ = 1) were mixed before infiltration. The RNA silencing suppression assay was carried out as described previously by Silhavy *et al.* (2002). Visual detection of GFP fluorescence was performed using a 100 W hand-held long-wave ultraviolet lamp (UV products, Upland, CA 91786, Black Ray model B 100AP).

Plasmid constructs

The infectious cDNA clones of CymRSV (Dalmay *et al.*, 1993), Cym19stop mutant (Szittya *et al.*, 2002) and 35S-GFP plasmid were described previously (Silhavy *et al.*, 2002). Primers corresponding to the first 20 nucleotides of 5′ and 3′ ends of GFP were used to amplify GFP from 35S-GFP, and cloned to *Eco*RV-digested Bluescript SK(+) creating SK-GFP. SK-GFP was digested with *Hpa*I and *Sma*I, dephosphorylated and then a full-length GFP PCR product was cloned into this vector in inverted orientation to construct SK-dsGFP. *Kpn*I and *Bam*HI fragments were cloned from SK-dsGFP to Bin61S to create 35S-dsGFP.

Protoplast preparation and inoculation

Protoplasts were isolated from *N.benthamiana* plants and transfected with *in vitro*-synthesized transcripts of genomic RNAs using the polyethylene glycol method as described previously (Dalmay *et al.*, 1993). After infection protoplasts were incubated in Versatile Enviromental Test Chambers (Sanyo, Tokyo Japan) under a 14 h light (50 µE m^{-2}s^{-1}) and 10 h dark regime at different temperatures.

In vitro RNA transcription and plant inoculation

In vitro transcription of CymRSV and Cym19stop, and inoculation of RNA transcripts onto *N.benthamiana* plants were performed as described previously (Dalmay *et al.*, 1993).

RNA gel blot analysis

Total RNA extraction and RNA gel blot analysis of high and low molecular weight RNAs was carried out as described previously (Szittya *et al.*, 2002). PCR fragments labelled with random priming method were used for northern analyses of high molecular weight RNAs. Radioactively labelled *in vitro* transcripts corresponding to the negative strand of virus RNA and antisense strand of GFP were used as probes for northern analyses of low molecular weight RNAs. For miRNA detection, 5′-labelled antisense DNA oligonucleotides corresponding to the

analysed miRNAs were used as probes. Labellings were carried out according to Sambrook *et al.* (1989).

In situ RNA hybridization

Upper leaves of systemically infected plants were fixed and paraffin embedded according to Jackson (1992). Hybridization probe to detect target RNA by *in situ* hybridization was prepared as described previously (Aranda *et al.*, 1996). Negative-sense RNA probe corresponding to the last 900 nucleotides of viral genomic RNA was prepared after linearizing the pBluescript II vector containing the target sequence with *Sma*I and transcribing with T7 RNA polymerase. Digoxigenin-11-UTP-labelled probe was hybridized to leaf cross-sections of 10 µm thickness and detected with alkaline phosphatase-conjugated anti-digoxigenin antibody, as described previously (Wang and Maule, 1995).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Note added in proof

During the review process of this paper, we were informed that temperature-dependent accumulation of siRNAs derived from inverted repeat transgenes was observed and reported by Kalantidis *et al*. (*Mol. Plant Microbe Interact*., 2002, **8**, 826–833).