Base-pair opening dynamics of the microRNA precursor pri-miR156a affect temperature-responsive flowering in Arabidopsis

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Article info

Article history:
Received 31 January 2017
Accepted 31 January 2017
Available online 2 February 2017

Keywords:
MicroRNA biogenesis
Flowering
NMR
Base-pair opening dynamics
RNA

Abstract

Internal and environmental cues, including ambient temperature changes, regulate the timing of flowering in plants. Arabidopsis miR156 represses flowering and plays an important role in the regulation of temperature-responsive flowering. However, the molecular basis of miR156 processing at lower temperatures remains largely unknown. Here, we performed nuclear magnetic resonance studies to investigate the base-pair opening dynamics of model RNAs at 16°C and investigated the in vivo effects of the mutant RNAs on temperature-responsive flowering. The A9C and A10CG mutations in the B5 bulge of the lower stem of pri-miR156a stabilized the C15-G98 and U16-A97 base-pairs at the cleavage site of pri-miR156a at 16°C. Consistent with this, production of mature miR156 was severely affected in plants overexpressing the A9C and A10CG constructs and these plants exhibited almost no delay in flowering at 16°C. The A10G and A9AC mutations did not strongly affect C15-G98 and U16-A97 base-pairs at 16°C, and plants overexpressing A10G and A9AC mutants of miR156 produced more mature miR156 than plants overexpressing the A9C and A10CG mutants and showed a strong delay in flowering at 16°C. Interestingly, the A9AC mutation had distinct effects on the opening dynamics of the C15-G98 and U16-A97 base-pairs between 16°C and 23°C, and plants expressing the A9AC mutant miR156 showed only a moderate delay in flowering at 16°C. Based on these results, we propose that fine-tuning of the base-pair stability at the cleavage site is essential for efficient processing of pri-miR156a at a low temperature and for reduced flowering sensitivity to ambient temperature changes.

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1. Introduction

Plant microRNAs (miRNAs) are small non-coding RNAs that are usually 20–22 nucleotides (nt) long. They specifically bind to their target sequences with the help of the RNA-induced silencing complex and either cleave the target gene mRNA or inhibit target gene translation [1]. The primary transcripts of miRNAs (pri-miRNAs) are transcribed from MIR genes and the resulting transcripts likely form hairpin structures composed of a terminal loop, an upper stem, a miRNA/miRNA* duplex, and a lower stem (see Fig. 1A for the structure of pri-miR156a). The pri-miRNAs are processed by the complex of DICER-LIKE 1 (DCL1), HYPONASTIC LEAVES1 and SERRATE to generate the mature miRNA in plants [2]. After HUA ENHANCER 1 methylates the released miRNA/miRNA* duplex, the duplex is exported to the cytoplasm [3,4]. One functional strand of the miRNA/miRNA* duplex is loaded into the ARGONAUTE complex [5] and the functional strand guides the complex to its target sequences that have perfect or partial sequence complementarity.

The secondary structure of pri-miRNAs plays an important role in miRNA processing in plants. A mutation that closes a bulge near a cleavage site in the lower stem of pri-miR171 resulted in decreased levels of mature miR171 [6]. For miR390a, a mutation 4–6 nt below the miR390a/miR390a* duplex affected the efficiency and accuracy of miR390a processing [7]. A ~15-nt segment in the lower stem in pri-miR172a is essential for pri-miR172a processing [8,9]. In contrast, the conserved upper stem, including a terminal loop, plays a crucial role in miR319 processing [10]. Therefore, the sequence and structure of pri-miRNAs contain information for miRNA processing.
Plants regulate the timing of the transition from the vegetative to the reproductive phase in response to internal and environmental cues. Even small changes in ambient temperature affect the plant floral transition and miRNAs play an important role in the regulation of temperature-responsive flowering [11-13]. MiR156a regulates ambient temperature-responsive flowering, negatively regulating SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 (SPL3). SPL3 binds to the FLOWERING LOCUS T promoter and promotes the floral transition in response to ambient temperature changes [12]. The importance of the secondary structure of pri-miR156a in the regulation of temperature-responsive flowering has been examined by analyzing structural mutations introduced into the pri-miR156a hairpin [14-16]. In plants overexpressing mutated pri-miR156a constructs, all mutations introduced in the upper stem of pri-miR156a reduced the levels of mature miR156, and caused a less-severe phenotype compared with the un-mutated construct. Mutations close to the miR156a/miR156a* duplex caused a stronger phenotype [14]. The second stem near the first cleavage site of pri-miR156a and pri-miR172a is important in the temperature response: mutations introduced in the second stem affected miRNA processing at 23 °C but not at 16 °C [14]. The structure of the lower stem of pri-miR156a also has a role in pri-miR156a processing. Mutations stabilizing the bulge below miR156a/miR156a* duplex affected base-pairing stability of second cleavage site of pri-miR156a, and affected miR156 processing at 23 °C [15]. Although the structural determinants for miR156 processing at 23 °C have been reported, the molecular basis of miR156 processing at lower temperatures remains largely unknown.

Here, we performed nuclear magnetic resonance (NMR) studies to investigate base-pair opening dynamics using model RNAs of wild-type (WT) and B5-stabilizing mutant constructs at 16 °C. We determined the levels of mature miR156 and miRNA processing in plants overexpressing WT pri-miR156a and mutant constructs to investigate the in vivo effects of the mutation at a low temperature. Our study revealed that B5-stabilizing mutations of pri-miR156a differentially affected base-pairing stability at the cleavage site and these changes of stability affected pri-miR156a processing and flowering time phenotypes at 16 °C.

2. Materials and methods

2.1. Sample preparation for NMR

The RNA oligomers were purchased from M-biotech Inc. (Korean
branch of IDT Inc., USA). The RNA oligomers were purified by reverse-phase HPLC and desalted using a Sephadex G-25 gel filtration column. The RNA samples were dissolved in 90% H2O/10% D2O aqueous solution containing 10 mM Tris-d11-HCl (pH 8.00 at 24.2 °C) and 50 mM NaCl. The Tris-HCl concentration was increased from 10 mM to 200 mM by successive additions of a 500 mM Tris-d11-HCl stock solution. The pH of the sample dissolved in Tris-HCl buffer was calculated using the equation ΔpKs = −0.031 × ΔT [17].

2.2. NMR experiments

All NMR experiments were performed on an Agilent DD2 700 MHz spectrometer (GNU, Jinju, Korea) or a Bruker Avance-III 800 MHz spectrometer (KBSI, Ochang, Korea) equipped with a cryogenic triple-resonance probe. One-dimensional (1D) NMR data were processed with either VNMR J (Agilent, CA, USA) or FELIX2004 (FELIXNMR, CA, USA), whereas 2D data were processed with NMRIPIPE [18] and analyzed with Sparky [19]. The exchange rates (kex) of the imino protons were determined as previously described [20,21].

2.3. Hydrogen exchange theory

The formalism of base-catalyzed proton exchange has been extensively described [15,20–22]. The exchange time (τex = 1/kex) for the base-paired imino proton is represented by Eqn. (1):

\[
\frac{1}{\tau_{ex}} = \frac{1}{\tau_0} + \frac{1}{K_{op}} \left( \frac{k_B [B]}{[I]} + \frac{1}{k_{int}} \right)
\]

(1)

where \(K_{op} (= k_{op}/k_i)\) is the equilibrium constant for base-pair opening, \(k_B\) and \(k_i\) are the rate constants for base-pair opening and closing, respectively, \(\tau_0\) is the base-pair lifetime (= 1/k_{op}), \(k_B\) is the rate constant for imino proton transfer by a base catalyst, \(k_{int}\) is the exchange rate constant catalyzed by an intrinsic base, and \([B]\) is the base catalyst concentration. Curve fitting the \(\tau_{ex}\) values of the imino protons as a function of the Tris (base form) concentration with Eqn. (1) gives the \(K_{op}\), \(\tau_0\) and \(k_{int}\) values. The lifetime for base-pair opening (\(\tau_{open} = 1/k_{cl}\)) is calculated using the relation \(\tau_{open} = \tau_0\).

The Gibbs free energy difference (ΔG_{bp}) between the closed and open states is calculated from the \(K_{op}\) value for base-pair opening using Eqn. (2):

\[
\Delta G_{bp}^0 = -\Delta G_{open}^0 = RT \ln(K_{op})
\]

(2)

where \(\Delta G_{open}^0\) is the Gibbs free energy change in the opening process, \(T\) is the absolute temperature and \(R\) is the universal gas constant. The differences in activation energies for base-pair opening (\(\Delta G_{op}^0\)) and closing (\(\Delta G_{cl}^0\)) in WT and mutant RNAs are calculated using Eqns. (3) and (4), respectively:

\[
\Delta G_{op}^0 = RT \ln\left( \frac{\tau_{mut}/\tau_{WT}}{k_{op}} \right)
\]

(3)

\[
\Delta G_{cl}^0 = RT \ln\left( \frac{\tau_{open,mut}/\tau_{open,WT}}{k_{cl}} \right)
\]

(4)

where the subscripts, WT and mut, indicate the thermodynamic parameters of the WT and mutant RNAs, respectively.

2.4. Plant materials, measurement of leaf numbers and temperature response

Arabidopsis thaliana Columbia-0 (Col-0) was used for transformation. The constructs for WT pri-miR156a and B5-stabilizing mutants were prepared as previously described [15]. The plants were grown in soil at 16 °C under long-day conditions (16 h light, 8 h dark) with a light intensity of 120 μmol/m²/s. The number of primary rosette and cauline leaves of homozygous plants at flowering was measured. We first scored the leaf numbers of primary transformants in the T1 generation and selected a few representative lines that showed leaf numbers close to the median value of leaf numbers seen in the T1 population at flowering to isolate homozygous lines. Thirty homozygous plants from each transgenic line were used to score leaf numbers. The distribution of leaf numbers is presented as a box plot [23], where center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles; dots represent individual data points.

The leaf number ratio (LNR) (number of leaves at flowering at 16 °C/number of leaves at 23 °C) was used as an indicator of ambient temperature-sensitivity [24,25]. Col-0 plants produce about 2-fold more leaves at 16 °C compared with 23 °C, therefore the LNR value of wild-type Col-0 plants is approximately 2.0. The LNR of completely ambient temperature-insensitive plants is 1.0; they produce identical numbers of leaves at different temperatures. Based on this criterion, plants showing LNR values greater than 2.0 were considered temperature-hypersensitive, whereas plants having LNR less than 2.0 were considered temperature-hyporesponsive.

2.5. MiRNA northern blot analysis

Eleven-day-old homozygous plants grown at 16 °C were pooled to extract total RNA using Plant RNA Purification Reagent (Invitrogen). Ten μg of total RNA was loaded onto 17% denaturing polyacrylamide gels containing 7 M urea and electrophoresed, then transferred to Hybond-NX neutral nylon membrane (GE Healthcare). The membrane was crosslinked with N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (Sigma) to increase detection sensitivity [26]. The hybridized membranes with probes labeled at the 3’ end with γ-32P ATP using OptiKinase (USB Corp., USA) were exposed and analyzed using Fuji BAS FLA-7000 (FUJI, Japan). U6 was used as an internal control. MiRNA northern blot experiments were performed in two biological replicates (independent samples harvested on different days) and one representative result is shown.

3. Results & discussion

3.1. Flowering time of plants overexpressing WT and mutant miR156a at 16 °C

To identify the effect of temperature on pri-miR156a processing at lower temperatures, we analyzed the flowering time, quantified as the leaf number at flowering, of plants overexpressing WT pri-miR156a (35S::miR156a) or B5-stabilizing mutant constructs (B5-A9C, B5-A9G, B5-A10C, and B5-A10GC) at 16 °C (Fig. 1B). At 16 °C, 35S::miR156a plants produced more leaves (52.8 leaves; approximately 2.5-fold) than the empty vector control (20.8 leaves) (Fig. 1C). Previously, we observed a ~1.7-fold increase in leaf number at 23 °C [15]. B5-A9C plants (28.3 leaves) and B5-A10GC plants (24.5 leaves) produced fewer leaves than 35S::miR156a plants at 16 °C, similar to plants grown at 23 °C [15]. In contrast, B5-A10C plants produced a similar number of leaves (50.1 leaves) to 35S::miR156a plants. At 16 °C, B5-A9G and B5-A9C plants produced significantly fewer leaves (45.0 leaves) compared to the 35S::miR156a and B5-A10C plants. Expression levels of mature miR156 in plants overexpressing B5-stabilizing mutant pri-miR156a constructs...
revealed that the levels were consistent with flowering time changes. As miR156 is a repressor of flowering, more miR156 likely delays flowering and thus plants have more leaves at flowering [12]. The B5-A10G and B5-A9AC plants, which produced more leaves, also accumulated more miR156 (2.6- and 2.7-fold increases, respectively) than B5-A9C and B5-A10CG plants (1.2- and 1.8-fold increases, respectively) (Fig. 1D). This suggested that among B5-stabilizing mutations A9C and A10CG mutations caused reduced production of mature miR156 and earlier flowering than WT pri-miR156a at 16 °C.

Next, we determined the leaf number ratios (LNR) to test whether the different processing of pri-miR156a caused different responses to ambient temperature, counting leaf numbers at 16 °C and using our previously measured data for 23 °C [15]. The LNR values of EV and 3SS::miR156a plants were 1.90 and 2.54, respectively, indicating that miR156a overexpression caused hypersensitivity to ambient temperature changes (Fig. 1E). The LNR of almost all transgenic plants was lower than that of 3SS::miR156a plants, indicating that mutations in the B5 bulge of pri-miR156a caused decreased sensitivity to differences in ambient temperature. Interestingly, B5-A9C, B5-A9AC, and B5-A10CG plants showed LNR values of 1.6 to 1.7, suggesting that these mutations strongly decreased sensitivity to ambient temperature. By contrast, the LNR value of B5-A10G plants (2.06) was intermediate between 3SS::miR156a plants and plants overexpressing other mutants. These observations suggested that the B5 bulge plays a role in miR156 processing at different ambient temperatures.

### 3.2. Base-pair opening dynamics of WT and mutant pri-miR156a constructs

The equilibrium constants for base-pair opening ($K_{op}$) and base-pair lifetimes ($t_0 = 1/k_{op}$) for the C15-G98 and U16-A97 base-pairs in the WT and mutant pri-miR156a constructs were determined from Tris-catalyzed imino proton exchange measurements at 16 °C. The $K_{op}$ values for C15-G98: (5.3 ± 0.2) × 10^{-6}; U16-A97: (11.1 ± 0.2) × 10^{-6} and $t_0$ values (C15-G98: 21.9 ± 0.7 ms; U16-A97: 2.3 ± 0.2 ms) in WT pri-miR156a were determined from analysis of the $\tau_{ex}$ data as a function of [Tris] using Eqn. (1) (Fig. 2A and B). Next, these data were used to calculate a lifetime for base-pair opening ($t_{open} = 1/k_{cl}$) (C15-G98: 117 ± 6 ns; U16-A97: 26 ± 2 ns) using the equation $t_{open} = t_0 / K_{op}$.

In A9C pri-miR156a, where the A9:G105 wobble pair in the B5 bulge is changed to a C9-G105 Watson-Crick base-pair, the C15-G98 and U16-A97 base-pairs have 4- and 3-fold smaller $K_{op}$, respectively, compared to WT pri-miR156a (Fig. 2C and D). Similar results were observed for the A10CG pri-miR156a, where two C10′-G104 and G10-C103 Watson-Crick base-pairs are introduced.

![Fig. 2](image-url) Hydrogen exchange times ($\tau_{ex} = 1/k_{ex}$) for the (A) G98 and (B) U16 imino protons as a function of the Tris concentrations at 16 °C. The solid lines are the best fits to Eqn. (1). The $K_{op}$, $t_0$, and $t_{open}$ values for (C) the C15-G98 and (D) U16-A97 base-pairs determined by the Tris-catalyzed NMR exchange experiments at 16 °C (this study) and 23 °C [15].
into the B5 bulge (Fig. 2C and D). In addition, these base-pairs have significantly shorter $r_0$ and $r_{\text{open}}$ values in these two mutants than WT pri-miR156a (Fig. 2C and D). From a previous study [15], these two mutants displayed the same pattern of base-pair opening dynamics at 23 °C (Fig. 2C and D). In A10G pri-miR156a, where the A10:C103 wobble pair in B5 is changed to a G10:C103 Watson-Crick base-pair, there were no significant effects on the opening dynamics of these two base-pairs both at 16 °C and 23 °C (Fig. 2C and D).

Of particular interest, the A9AC pri-miR156a, where the C9'-G104 Watson-Crick base-pair is newly introduced into the B5 bulge (Fig. 1B), displayed a distinct pattern of base-pair opening dynamics at 16 °C, compared to at 23 °C. The C15-G98 and U16-A97 base-pairs of the A9AC mutant had 3- and 2-fold smaller $k_{\text{op}}$ values, respectively, compared to WT pri-miR156a at 16 °C, whereas these base-pairs showed no significant effects on the base-pair opening dynamics at 23 °C, like the A10G mutant (Fig. 2C and D).

3.3. Base-pair opening thermodynamics of WT and mutant pri-miR156a constructs

The Gibbs free energy differences between the closed and open states ($\Delta G_{\text{op}}$) of the base-pairs were calculated from $k_{\text{op}}$ using Eqn. (2). In WT pri-miR156a, the $\Delta G_{\text{op}}$ values of the C15-G98 (−6.98 ± 0.04 kcal/mol) and U16-A97 (−6.55 ± 0.02 kcal/mol) base-pairs at 16 °C are similar to the data at 23 °C (C15-G98: −6.87; U16-A97: −6.35 kcal/mol) reported in a previous study [15].

Similar to 23 °C, the differences between the $\Delta G_{\text{op}}$ values of the C15-G98 and U16-A97 base-pairs in the A10G and WT pri-miR156a ($\Delta G_{\text{op}}$) were only 0.08 and 0.11 kcal/mol, respectively, at 16 °C (Fig. 3). However, in the A9C and A10CG pri-miR156a, the $\Delta G_{\text{op}}$ values of the C15-G98 and U16-A97 base-pairs were −0.9 to −0.6 kcal/mol at both 16 °C and 23 °C (Fig. 3). These results indicated that the A9C and A10CG mutations on the B5 bulge led to greater stabilization of the C15-G98 and U16-A97 base-pairs, but the A10G mutation had little effect on these base-pair stabilities.

The $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ values represent differences in the Gibbs free energy between the closed and transition states, and the open and transition states, respectively. The differences in $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ between the mutant and WT pri-miR156a ($\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$) were calculated from $k_{\text{op}}$ and $k_{\text{cl}}$ using Eqs. (3) and (4). In the A9C and A10CG mutants, the C15-G98 and U16-A97 base-pairs have more negative $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ values than WT pri-miR156a at both 16 °C and 23 °C (Fig. 3). However, the A10G mutations had little effect on the activation energies for base-pair opening and closing at both temperatures (Fig. 3).

The A9AC mutant displayed a distinct pattern of base-pair stabilities and opening dynamics between 16 °C and 23 °C. The C15-G98 base-pair in the A9AC mutant showed greater stabilities ($\Delta G_{\text{op}}$: −0.62 kcal/mol) and faster opening dynamics ($\Delta G_{\text{cl}}$: −0.83 kcal/mol) than WT pri-miR156a at 16 °C, while the corresponding $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ values at 23 °C were 0.01 and 0.06 kcal/mol, respectively (Fig. 3A). Similarly, the U16-A97 base-pair had much more negative $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ values at 16 °C compared to 23 °C (Fig. 3B). However, other three mutants showed a similar pattern of the base-pair stabilities and opening dynamics at both 16 °C and 23 °C (Fig. 3).

3.4. Biological implications

Our study revealed that B5-stabilizing mutations of pri-miR156a changed base-pairing stabilities at the cleavage site and such changes in stability affected miR156a processing and leaf production phenotypes at a low temperature.

The plants overexpressing B5-stabilizing mutants at 16 °C, except B5-A9AC plants, showed a flowering time phenotype, similar to that seen at 23 °C. The B5-A9C and B5-A10CG plants flowered with fewer leaves than the 35S::miR156a plants (Fig. 1C). B5-A10G plants flowered with a similar number of leaves with 35S::miR156a plants at 16 °C, as seen at 23 °C. However, the B5-A9AC plants produced slightly fewer leaves than 35S::miR156a plants. These leaf numbers at flowering of plants overexpressing B5-stabilizing

Fig. 3. Schematic representations of the Gibbs free energy diagram of the base-pair opening and closing of the C15-G98 (A) and U16-A97 (B) base-pairs in the WT (black), A9C (red), A9AC (purple), A10G (blue) and A10CG (green) pri-miR156a at 16 °C (left, this study) and 23 °C (right, [15]). Gray arrows indicate the $\Delta G_{\text{op}}$ values of WT pri-miR156a. Red and purple arrows indicate differences in $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ between the WT and A9C pri-miR156a, respectively. Gray arrows indicate $\Delta G_{\text{op}}$ values of WT pri-miR156a. The $\Delta G_{\text{op}}$ and $\Delta G_{\text{cl}}$ values at 16 °C (this study) and 23 °C [15] are shown at right in each figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
mutants at 16°C are consistent with their mature miR156a levels, suggesting that B5-stabilizing mutations affected the efficiency of miR156 processing at 16°C. As the base-pair stabilities at the cleavage site of pri-miR156a at 16°C increased, the levels of mature miR156 and leaf number at flowering also increased. The B5–A9C and B5–A10C plants showed decreased levels of mature miR156 and flowered with fewer leaves than 35S::miR156a plants (Fig. 1C and D). These mutations induced greater stabilization of base-pairs at the cleavage site of pri-miR156a. The ΔΔGopening values of the C15–G98 and U16–A97 base-pairs of A9C mutation were −0.86 and −0.61 kcal/mol, respectively (Fig. 3). Moreover, the C15–G98 and U16–A97 base-pairs had significantly lower activation energies for base-pair opening, with ΔΔGopening of −1.69 and −1.09 kcal/mol, respectively (Fig. 3). Similar results were observed for the A10C mutant (Fig. 3). In contrast, the A10C mutation did not significantly affect the stabilities and opening dynamics of the C15–G98 and U16–A97 base-pairs at 16°C. The plants overexpressing this mutant showed similar or lower mature miR156 levels (Fig. 1D) and similar numbers of leaves as observed for the plants overexpressing this mutant showed similar or lower mature miR156 levels (Fig. 1D) and similar numbers of leaves as observed for the

In summary, we investigated the in vivo effects of pri-miR156a on temperature-responsive flowering and examined base-pair opening dynamics using model RNAs at 16°C. The stabilities of the C15–G98 and U16–A97 base-pairs at the cleavage site reflected the mature miR156 levels as well as miR156-driven flowering time phenotypes, which are affected by mutations at the B5 bulge. The B5-stabilizing mutations displayed unique patterns of base-pair stabilities at the cleavage site and thus induced distinct effects on miR156 processing at 16°C and 23°C. Our study suggested that the base-pair-opening dynamics at the cleavage site are essential for the efficient processing of pri-miR156a and regulation of flowering in response to ambient temperature.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grants [2012R1A1A027750 (BRL) to J.-H.L.; Creative Research Initiative Program (2008-0061988) to J.-H.L.'s work for performing the NMR experiments] and Young Ja Kim for technical assistance.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.01.185.

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