Molecular insights into plant cell proliferation disturbance by *Agrobacterium* protein 6b

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The *Agrobacterium* Ti plasmid (T-DNA) 6b proteins interact with many different host proteins implicated in plant cell proliferation. Here, we show that *Arabidopsis* plants overexpressing 6b display microRNA (miRNA) deficiency by directly targeting SERRATE and AGO1 via a specific loop fragment (residues 40–55). In addition, we report the crystal structures of *Agrobacterium tumefaciens* AK6b at 2.1 Å, *Agrobacterium vitis* AB6b at 1.65 Å, and *Arabidopsis* ADP ribosylation factor (ARF) at 1.8 Å. The 6b structure adopts an ADP-ribosylating toxin fold closely related to cholera toxin. In vitro ADP ribosylation analysis demonstrates that 6b represents a new toxin family, with Tyr 66, Thr 93, and Tyr 153 as the ADP ribosylation catalytic residues in the presence of *Arabidopsis* ARF and GTP. Our work provides molecular insights, suggesting that 6b regulates plant cell growth by the disturbance of the miRNA pathway through its ADP ribosylation activity.

[**Keywords:** ADP ribosylation toxin; *Agrobacterium* protein 6b; crystal structure; miRNA machinery; miRNA phenotype]

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Agrobacterium tumefaciens cells that harbor Ti plasmid (T-DNA) induce crown gall tumors on dicotyledonous plants. Among the T-DNA-encoded proteins, 6b appears to play a role in the expression of plant genes related to cell proliferation and the modification of crown gall morphology, although the 6b protein itself is not required for the formation of crown galls (Garfinkel et al. 1981; Tinland et al. 1992).

The 6b genes have remarkable and unique effects on plant growth. They induce tumors on stems in a limited number of plant species, including *Nicotiana glauca* (Hooykaas et al. 1988; Tinland et al. 1989; Kitakura et al. 2002), grapevine (Huss et al. 1990), and *Nicotiana rustica* (Garfinkel et al. 1981; Paulus et al. 1991); increase wound-induced division of cells in *N. rustica* (Tinland et al. 1992); promote *iaa*- and *ipt*-induced cell division (Hooykaas et al. 1988; Tinland et al. 1989); and enhance tumor formation or increase the size of crown galls induced on *N. rustica* through the action of the *auxin* genes and the *cytokinin* gene (Tinland et al. 1989). Expression of 6b genes also elicits morphological and anatomical changes in tobacco plants, and modifies

cotyledons, flowers, petioles, stems, and roots (Tinland et al. 1990, 1992; Helfer et al. 2003; Gremillon et al. 2004). Transgenic 6b tobacco plants develop enations, catacollas, tubular leaves, fragmented leaf primordia, hyponastic petioles, and epinastic leaf veins (Clément et al. 2006).

The predicted amino acid sequence of 6b is distantly related to the RolB family proteins, whose expression also results in aberrant growth and abnormal morphology of both roots and shoots (Spena et al. 1987; Levesque et al. 1988; Stieger et al. 2004). Protein 6b localizes to the nucleus and was reported to bind to several nuclear proteins—such as NtSIP1 (*Nicotiana tabacum* Six-b-interacting protein 1) (Kitakura et al. 2002), NtSIP2 (Kitakura et al. 2008), and histone H3 (Terakura et al. 2007)—and affects the expression of various plant genes in transgenic *Arabidopsis*. Transgenic *Arabidopsis* plants expressing the AK6b gene display leaf abnormality at early developmental stages, such as upward curling and serration of leaves, protrusion on the abaxial side of leaves, altered flower structure, and callus formation on hormone-free medium.

Much effort has been expended to elucidate 6b functions at the cellular and molecular levels. So far, different hypotheses have been proposed to explain the effects of 6b on the growth of plant cells. The 6b gene could possess auxin-like and cytokinin-like activity by modulating auxin or cytokinin receptors, and promote activity of genes involved in auxin and cytokinin biosynthesis

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(Wabiko and Minemura 1996; Clément et al. 2006). *6b* might induce plants to synthesize a phloem-mobile growth factor (enation factor) (Helfer et al. 2003). In addition, the *6b* gene has also been proposed to modulate the metabolism of phenolic compounds (Gális et al. 2002, 2004; Kakiuchi et al. 2006); induce changes in gene expression, resulting in phenylpropanoid changes (Kitakura et al. 2002; Terakura et al. 2006); modify osmotic controls required for normal and wound-induced cell expansion (Clément et al. 2006); and cause the absorption, retention, and abnormal accumulation of sugars (Clément et al. 2007).

Recent studies on *6b* further suggest that *6b* might affect chromatin status as a chromatin chaperon or affect the expression of class I *KNOX*, *CUC*, *IAA*, and cell cycle-related genes as a transcriptional regulator of plant cell proliferation in tobacco and *Arabidopsis* (Terakura et al. 2006, 2007).

Although much work has been done on *6b*, the exact functional role of *6b* at the molecular level remains unknown owing to the lack of structural motif information obtained from *6b* sequences. To investigate the molecular mechanism of leaf abnormality elicited by *6b* overexpression in plants, we report here that *Arabidopsis* plants overexpressing *6b* display a microRNA (miRNA) deficiency phenotype. This phenotype can be attributed to the targeting of the miRNA pathway by targeting the miRNA processing machinery and slicing machinery by *6b*. In addition, we also report high-resolution crystal structures of AK6b, AB6b, and *Arabidopsis* ADP ribosylation factor (ARF). Our data demonstrate that *6b* adopts an ADP ribotransferase fold quite close to that of cholera toxin and displays ADP ribosylation activity in the presence of *Arabidopsis* ARF and GTP. However, *6b* has a completely different set of residues within the substrate-binding pocket for ADP ribosylation activity.

Results

6b interferes with miRNA pathways in *Arabidopsis*

Many plant viruses are known to encode proteins that suppress RNA silencing and interfere with the miRNA pathway (Zhang et al. 2006b; Baumberger et al. 2007; Bortolamiol et al. 2007). Since T-DNA can be considered a DNA virus, we speculated that the *6b* protein may also interfere with the miRNA pathway in *Arabidopsis*. Careful comparison of the morphological phenotypes of *35S::AK6b* overexpression in plants with *ago1-27* and *se-1* mutant lines showed that these transgenic plants partially phenocopied these two miRNA-deficient mutants, which have strongly serrated leaves (Fig. 1A). This observation prompted us to investigate the expression levels of several miRNAs involved in leaf development, and their target mRNAs. Along with the severe morphological phenotype accompanying the overexpression of *35S::AK6b* in *Arabidopsis*, miR162, miR164, and miR319 were significantly decreased, whereas miR165/166 was slightly decreased (Fig. 1B, left panel). On the other hand, the mRNA targets of these miRNAs accumulated to a high level, showing an inverse correlation with the low expres-

Figure 1. Expression of miRNA in *AK6b* transgenic plants. (A) Comparison of the morphological phenotype between *35S::AK6b*, *ago1-27*, and *se-1* plants. Photographs were taken for the second, third, and fourth true leaves of 4-wk-old seedlings. Bar, 1 cm. (B) Accumulation of small RNAs and target mRNAs in wild-type (*Col-0*), *se-1*, and *35S::AK6b* plants. Each lane contained 12 μ g of RNA. rRNAs were used as a loading control. For analysis of target mRNAs, quantifications of each cDNA sample were made in triplicate, and consistent results from at least two independent RNA samples were used.

sion level of the individual miRNAs (Fig. 1B, right panel). Notably, the molecular miRNA-deficient phenotypes of *35S::AK6b* plants and the *se-1* mutant only partially overlapped. For example, the expression level of miR319 was significantly decreased in *35S::AK6b* plants instead of the *se-1* mutant (Fig. 1B, left panel). In contrast, the expression level of miR165/166 was significantly decreased in the *se-1* mutant instead of *35S::AK6b* plants (Fig. 1B, left panel). Similarly, the accumulation of their target mRNAs—such as *TCP4* (miR319 target) and *REV* (miR165/166 target)—in *35S::AK6b* plants and the *se-1* mutant was inversely correlated to the individual miRNA expression level (Fig. 1B, right panel).

6b targeting *Arabidopsis* AGO1 and Serrate (SE) *in vitro* and *in vivo*

The partially overlapping miRNA deficiency phenotypes among the *35S::AK6b* plants, *se-1* mutant, and *ago1-27* mutant prompted us to test the possible direct interactions between AK6b, SE, and AGO1. We performed *in vitro* pull-down assays using various purified protein fragments to examine whether the AK6b-AGO1 or AK6b-SE interaction was direct, and attempted to map the interaction domains. We systematically generated six truncated AGO1

mutants from the N terminus (NT) to the C terminus (CT). In vitro pull-down assays showed that the AGO1-NT fragment (residues 185–371) was the primary interaction domain targeted by AK6b (Fig. 2A). This fragment corresponds to the entrance channel for the small RNA and its target mRNA (Fig. 2B). To investigate the direct interaction between 6b and SE, we generated two truncated SE fragments from the N terminus to the C terminus. The in vitro pull-down assay showed that both the SE-NT fragment (residues 194–240) and the SE-Mid fragment (residues 241–469) were targeted by AK6b (Fig. 2C,D). To investigate whether 6b interacts with SE and AGO1 in vivo, we performed coimmunoprecipitation (co-IP) experiments using extracts prepared from *35S::AK6b* plants. We found that the SE antibody indeed detected a band in the immunocomplex pulled down by the AK6b antibody (Fig. 2E, bottom middle panel). Similarly, we were able to coimmunoprecipitate endogenous AGO1 by the AK6b antibody from the extracts of *35S::AK6b* plants (Fig. 2E,

bottom panel). The specificity of the anti-AtAGO1 antibody is shown in Supplemental Figure S1.

6b targeting *Arabidopsis* Histone H3 in vitro

To confirm whether 6b has a broad spectrum of targets beyond the miRNA machineries and interacts directly with *Arabidopsis* Histone H3, we performed an in vitro pull-down assay by immobilizing GST-AK6b or GST-AB6b on the GST beads and incubating His-SUMO-H3 with the bound GST-AK6b or GST-AB6b protein. As expected, both GST-AK6b and GST-AB6b were able to pull down His-SUMO-H3, whereas GST alone was not able to pull down His-SUMO-H3 (Supplemental Fig. S2, left panel).

Overall crystal structures of AK6b and AB6b

To investigate the molecular mechanism of 6b proteins on miRNA pathway disturbance, we successfully solved

the high-resolution structures of AK6b and AB6b. The crystal structure of the full-length *A. tumefaciens* AK6b at 2.1 Å was solved by multiple anomalous dispersion (MAD) phasing, whereas *Agrobacterium vitis* AB6b at 1.65 Å was determined by molecular replacement (using AK6b as the search model), with crystallographic statistics summarized in Table 1. AK6b and AB6b have very similar sequences and almost identical structures, except that AB6b harbors a little shorter repeating Glu residue loop in the middle. Therefore, hereafter we use the generic name 6b to represent both the AK6b and AB6b proteins and the amino acid residue numbers from AK6b.

6b contains nine β strands (β 1– β 9), three regular α helices (α 1– α 3), and two very short α helices (α 1'– α 2') (Fig. 3A), and possesses an α/β fold with five α helices packed against nine β strands at one surface, with an overall dimension of 50 Å $\times$ 35 Å $\times$ 25 Å. The 6b central module contains two perpendicular β sheets (β 1, β 2, β 5, β 8, and β 9 forming one group, and β 3, β 4, β 6, and β 7 forming another group) and one α helix (α 2) sitting on top

of these two β sheets, surrounded by several loops and two α helices (α 1 and α 3) (Fig. 3B).

A well-ordered loop (residues 113–134)—including the two very short α helices (α 1' and α 2')—connecting α 3 and β 5 forms a lid-like architecture covering the central structural module formed by the perpendicular β sheets. Another well-ordered 17-amino-acid-long loop (residues 40–55) connecting α 1 and β 1 extends from the central module of the structure. This loop participates in protein packing and may serve as a structural scaffold for protein–protein interaction. The well-studied loop with repeating Glu residues (10 Glu residues for AK6b and six Glu residues for AB6b) (Kitakura et al. 2002, 2008) is disordered in both AK6b and AB6b structures.

Target-binding loop

A comparison of the crystal packing of AK6b and AB6b revealed one conserved protein–protein interaction loop, which is unique to this protein family. In the AK6b

Table 1. Data collection, phasing, and refinement statistics

Data collection		AK6b		AB6b	AtARF
Space group		P3 ₂ 21		P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Molecules/ASU		3		2	2
Data set	Peak	Inflection	Remote	Native	Native
Cell dimensions					
<i>a</i>		80.458 Å		79.366 Å	88.710 Å
<i>b</i>		80.458 Å		89.926 Å	89.977 Å
<i>c</i>		245.708 Å		59.583 Å	46.049 Å
Wavelength	0.9790 Å	0.9794 Å		1.1 Å	1.075 Å
Resolution ^a	50~2.1 Å	50~2.1 Å	50~2.1 Å	50~1.65 Å	50~1.8 Å
	(2.18~2.10 Å)	(2.18~2.10 Å)	(2.18~2.10 Å)	(1.68~1.65 Å)	(1.83~1.80 Å)
R _{sym}	7.4% (35.4%)	7.4% (36.0%)	8.2% (42.3%)	7.3% (52.1%)	5.0% (24.4%)
I/ σ (I)	30.3/2.8	27.9/2.7	22.8/1.3	44.8/4.0	55.9/11.4
Completeness ^a	81.1% (32.9%)	74.6% (21.8%)	66.0% (12.9%)	99.9% (99.6%)	99.8% (100%)
Redundancy	9.7 (4.4)	9.7 (3.7)	7.7 (7.3)	13.9 (10.5)	14.1 (14.5)
Initial figure of merit		0.5			
Refinement					
Resolution range	50~2.1 Å			50~1.65 Å	50~1.80 Å
Number of reflections	43,896			49,396	33,083
R _{work} /R _{free}	20.2%/23.7%			17.8%/20.6%	24.1%/19.9%
Number of atoms					
Protein	4,588			3,046	2,665
Water	258			342	290
GDP					2
Mg ²⁺					2
B factors					
Protein	39.8 Å ²			17.7 Å ²	27.6 Å ²
Water	38.7 Å ²			34.2 Å ²	32.9 Å ²
GDP					18.4 Å ²
Mg ²⁺					16.4 Å ²
Root-mean-square deviations					
Bond lengths	0.010 Å			0.009 Å	0.015 Å
Bond angles	1.18°			1.15°	1.50°
Percent favored (disallowed) in Ramachandran plot	90.3% (0%)			92.4% (0%)	93.7% (0%)

^aValues for the highest-resolution shell are in parentheses.

Figure 3. Sequence alignment and overall structure of *Agrobacterium* protein 6b. (A) Sequence alignment and secondary structure of *Agrobacterium* protein 6b. The aligned sequences are in the order of *A. tumefaciens* AK6b, *A. vitis* AB6b, *A. vitis* T6b, and *Agrobacterium rhizogenes* Ro1b. The secondary structure diagram for AK6b is shown at the top. α Helices are colored in yellow, and β strands are in green. Conserved residues are shaded in cyan (80% similarity) and green (60% similarity), whereas essentially invariant residues are in yellow. Both the target-binding loop and the active site loop are indicated. The putative catalytic residues in 6b are highlighted by a black star below the sequence alignment. (B) Stereoview ribbon representation of AK6b with the target-binding loop colored in red and the active site loop colored in brown.

structure, one bulky extra density was observed sandwiched between the C-terminal loop region (residues 204–208) of 6b molecule 1 and one extended loop (residues 40–55) of 6b molecule 2 (Fig. 4A). Due to the high resolution of the AK6b structure (Supplemental Fig. S3, left panel), we were able to trace most of the NAD^+ molecule, including the ADP and the pyro-phosphate moieties, although no NAD^+ molecule was added into our crystallization buffers (Supplemental Fig. S3, right panel). It is possible that the NAD^+ molecule might be synthesized by *Escherichia coli* itself and formed a complex with 6b. Interestingly, PJ34, a competitive inhibitor of the NAD^+ molecule, was packed between two cholix toxin molecules in the crystal structure of the catalytic domain of the cholix toxin (Jørgensen et al. 2008b).

The conserved loop participating in the crystal packing and the protein–protein interaction in both the AK6b and AB6b crystal structures prompted us to test whether the 6b protein can self-associate, and whether this loop plays a role in the protein–protein interaction in vitro. In vitro pull-down assays showed that MBP-AK6b was able to pull down His-AK6b and His-AB6b (Fig. 4B, left panel). In contrast, MBP alone was not able to pull down His-AK6b

or His-AB6b (Fig. 4B, left panel). Similarly, GST-AK6b was able to pull down His-AK6b and His-AB6b (Fig. 4B, left panel), whereas GST alone was not able to pull down His-AK6b or His-AB6b (Fig. 4B, left panel). In vitro pull-down assays further demonstrated that this extended loop (residues 40–55) indeed participates in the protein–protein interaction, because MBP-AK6b ($\Delta 40$ –55) was not able to pull down His-AK6b, whereas either GST-AK6b ($\Delta 200$ –208) or MBP-AK6b ($\Delta 164$ –184) was able to pull down His-AK6b (Fig. 4B, right panel).

To investigate whether this extended loop (residues 40–55) also plays a role in SE binding, we performed an in vitro pull-down assay. Our assay confirmed that this extended loop (residues 40–55) also plays a decisive role for SE binding, because MBP-AK6b ($\Delta 40$ –55) was not able to pull down His-SE, whereas both GST-AK6b ($\Delta 200$ –208) and MBP-AK6b ($\Delta 164$ –184) were able to pull down His-SE (Fig. 4C).

To investigate whether this conserved loop also plays a general role for 6b to interact with its target proteins, such as *Arabidopsis* Histone H3, we performed an in vitro pull-down assay by immobilizing GST-AK6b on the GST beads or MBP-AK6b on the MBP beads and incubating His-SUMO-H3 with the bound GST-AK6b or MBP-AK6b protein. As expected, both GST-AK6b and MBP-AK6b were able to pull down His-SUMO-H3, whereas neither GST nor MBP alone was able to pull down His-SUMO-H3 (Supplemental Fig. S2). Similarly, in vitro pull-down assays further demonstrated that this extended loop (residues 40–55) indeed plays a decisive role for H3 binding, because MBP-AK6b ($\Delta 40$ –55) was not able to pull down His-SUMO-H3, whereas both GST-AK6b ($\Delta 200$ –208) and MBP-AK6b ($\Delta 164$ –184) were able to pull down His-SUMO-H3 (Supplemental Fig. S2).

To investigate whether this extended loop (residues 40–55) does play a significant role in vivo, we analyzed transgenic plants expressing 6b mutant derivatives. Although the severe morphological phenotypes displayed by *35S::AK6b* overexpression, such as dwarf stem and leaf serration, have been largely reverted to those of wild-type *Col-0* control in *35S::AK6b* ($\Delta 40$ –55) mutant plants, the protein expression levels of AK6b ($\Delta 40$ –55) were dramatically decreased beyond detectable levels (data not shown). Interestingly, the introduction of the similar deletion or point mutations at 6b overexpressed in *Nicotiana tabacum* and *Kalanchoe daigremontiana* also directly results in the significant decreased expression level of 6b protein in these host plants, suggesting that these key residues probably also play a role in protein stability (Helfer et al. 2002).

6b shares structural similarities with ADP-ribosylating toxins

To investigate the molecular mechanism for 6b-mediated gene regulation, a three-dimensional structure homolog search was performed by using the Dali server (<http://www.ebi.ac.uk/dali>). Surprisingly, the central module of the 6b structure, including the two perpendicular β sheets and the closely packed α helix ($\alpha 2$), shows close

with the addition of increasing amounts of TAD (Supplemental Fig. S4).

Putative NAD⁺-binding site of 6b

Similar to exotoxin A, the aromatic ring cage environment created by Tyr 121, Tyr 153, Tyr 156, and Tyr 121 probably participates in NAD⁺ binding by anchoring the nicotinamide ring of NAD⁺. Tyr 66 may also stabilize NAD⁺ binding by forming a hydrogen bond with the bound NAD⁺, whereas the Thr 93 side chain probably forms a hydrogen bond to the putative catalytic Y153 residue (Fig. 6B).

Similar to other bacterial ADP-ribosylating toxins, 6b structures also share two crucial features around the

Figure 5. AK6b adopts an ADP-ribosylating toxin fold. (A) Cartoon representation of the AK6b structure (left panel), exotoxin A structure (middle panel), and cholera toxin structure (right panel). The ADP ribotransferase core domain is colored in red, whereas the structural lid covering the active site is colored in orange. (B) Surface view of the AK6b structure (left panel), exotoxin A structure (middle panel), and cholera toxin structure (right panel). The view orientation is the same as in A.

NAD⁺-binding pocket: the active site loop and the ARTT motif (ADP-ribosylating toxin turn–turn motif), which consists of residues from two adjacent protruding turns (Fig. 6A; Deng and Barbieri 2008).

In the 6b structure, a long well-ordered loop (residues 113–134) occludes the putative NAD⁺-binding site, which must relocate to allow for substrate binding (Fig. 6A). This active site loop may act as an arm to recognize ADP-ribose acceptor substrates. Interestingly, the conserved STS motif, probably essential for NAD⁺ binding in most ADP-ribosylating toxins, is partially conserved at the AK6b sequence (¹²⁶STNS¹²⁹). Similar to other ADP-ribosylating toxins, adjacent to the putative STS motif, one highly conserved aromatic residue (Tyr 121) among all of the 6b sequences places its aromatic side chain into the putative NAD⁺-binding pocket, suggesting that this aromatic residue may participate in NAD⁺ binding (Fig. 6B).

In the 6b structure, a short loop (residues 148–151), together with the connected two anti-parallel β strands ($\beta 6$ and $\beta 7$), is aligned well with the ARTT motif conserved at both the exotoxin A and cholera toxin structures (Fig. 6A; Supplemental Fig. S5). However, instead of the conserved Glu–X–Glu (where X is any amino acid) motif as catalytic residues, 6b has two invariable Tyr residues (Tyr 153 and Tyr 156) probably functioning as the putative catalytic residues (Fig. 6A). In the 6b structure, the Tyr 153 side chain points toward the NAD⁺-binding pocket, whereas the Tyr 156 side chain points away from the NAD⁺-binding pocket, suggesting that Tyr 153 may play a primary catalytic role for ADP ribosylation.

Arabidopsis ARF is required for 6b function

Structural comparison of 6b with cholera toxin suggests that 6b proteins may represent a new toxin family and may display ADP ribosylation activity. To determine the putative ADP ribosylation of 6b in vitro, we performed an ADP-ribosylation assay by using in vitro expressed and

purified SE as the substrate, following the method described (Armstrong and Merrill 2001). However, ADP ribosylation experiments showed that neither AK6b nor AB6b alone displays ADP ribosylation activity (Fig. 7A, lane 1; data not shown). The closely structural features between 6b and cholera toxin prompted us to investigate whether 6b shares the similar feature of cholera toxin, which requires a host ARF for ADP ribosylation activity. A Blast search based on the human ARF6 sequence against the *Arabidopsis* protein database retrieved only one sequence (*Arabidopsis* ARF 1A1C) sharing high sequence similarity with human ARF6 (Supplemental Fig. S6). To investigate a possible direct interaction between 6b and *Arabidopsis* ARF, we generated N-terminal GST-fused AK6b protein and N-terminal His-fused *Arabidopsis* ARF protein. An in vitro pull-down assay showed that *Arabidopsis* ARF indeed interacts with AK6b (Fig. 7B).

To test whether *Arabidopsis* ARF indeed plays a crucial role for ADP ribosylation, we repeated the in vitro ADP ribosylation assay by the addition of *Arabidopsis* ARF together with GDP or GTP. As expected, AK6b displayed robust ADP ribosylation activity in the presence of *Arabidopsis* ARF and GTP (Fig. 7A, lane 4). We also introduced an alanine mutation at the putative catalytic residues identified from the crystal structures (Y66A, T93A, Y121A, and Y153A) to repeat the in vitro ADP ribosylation assay. All of these mutants displayed compromised ADP ribosylation activity (Fig. 7B, lane 5–8). In contrast, the D146A mutant with a mutation in a noncatalytic residue displayed ADP ribosylation activity similar to that of wild-type AK6b (Fig. 7B, lane 9).

To test whether AtAGO1 and/or SE is ADP-ribosylated by 6b in vivo, we coimmunoprecipitated the protein complex from protein extracts of 35S::AK6b plants by an anti-AK6b polyclonal antibody. Then we used an anti-ARF monoclonal antibody to detect whether ARF is indeed tightly associated with AK6b. As expected, a clear band corresponding to the molecular weight of ARF was

Figure 6. Putative active site of AK6b. (A) Overall view of putative active pockets of AK6b (left panel), exotoxin A (middle panel), and cholera toxin (right panel). The structure of the similar NAD⁺-binding domain is colored in red and other subdomains are in green, whereas the putative active site loop is in orange. Both the active site loop and ARTT loop are indicated. (B) Detailed view of putative active pockets of AK6b (left panel), exotoxin A (middle panel), and cholera toxin (right panel). Catalytic residues involved in NAD⁺ binding are indicated and are colored in yellow.

detected by Western blot (Fig. 7C). These experiments demonstrated that ARF is indeed the protein partner of 6b in vivo, and strongly support the notion that both SE and AGO1 are ADP-ribosylated by 6b in vivo.

To investigate whether these residues play significant roles for the miRNA-deficient phenotype displayed by 35S::AK6b plants, we performed a transgenic plant screen assay. Although the miRNA-deficient phenotype has been largely reversed at 35S::AK6b (Y66A, T93A, Y121A, or Y153A) mutants, the protein expression levels of AK6b mutants (Y66A, T93A, Y121A, or Y153A) in vivo were dramatically decreased beyond detectable levels (data not shown). In contrast, the 35S::AK6b (D146A) mutant showed a similar miRNA-deficient phenotype, and the protein expression level of the mutant in vivo was readily detectable (data not shown).

Crystal structure of Arabidopsis ARF resembles human ARF6 structure

To further investigate the molecular mechanism of ADP ribosylation catalyzed by 6b in the presence of ARF, we successfully determined the crystal structure of *Arabidopsis* ARF in complex with GDP at 1.8 Å, with crystallographic statistics summarized in Table 1. *Arabidopsis* ARF shares high structural similarity to human ARF6 (Fig. 7D). Similar to human ARF6, the N-terminal α helix of *Arabidopsis* ARF is positioned in the hydrophobic pocket opposite the GDP-binding site, whereas GDP-Mg²⁺ is deeply buried in the protein core and interacts with the switch regions (Fig. 7D, left panel). Therefore, a similar dramatic conformational change, including the rearrangements of the N terminus and switch regions triggered by GTP hydrolysis, should be expected for *Arabidopsis* ARF. The close structural similarity between *Arabidopsis* ARF and human ARF6 in complex with GDP (Fig. 7D, middle panel) suggests that *Arabidopsis* ARF in complex with GTP might adopt the similar conforma-

tion, as revealed by the human ARF6-GTP structure (Fig. 7D, right panel).

Discussion

6b directly targets miRNA machineries

Interference with RNA silencing by viral suppressors represents an anti-host defense response by viral pathogens (for review, see Yang and Yuan 2009). T-DNAs can be considered to be functionally related to a DNA virus. The observation that 35S::AK6b plants display a morphological phenotype similar to that of 35S::FNY2b plants raises the question of whether protein 6b functions as an RNA silencing suppressor (Zhang et al. 2006b). The partially overlapping morphological and molecular phenotypes between 35S::AK6b plants and ago1-27 and se-1 mutant plants further suggest that 6b probably interferes with miRNA pathways (Fig. 1). Our results show that AK6b probably executes its suppressor function by directly targeting SE and AGO1, two key components of the miRNA machineries in *Arabidopsis* (Fig. 2). We also mapped the primary targeting region on AGO1 to the N-terminal domain adjacent to the RNA entrance groove and the primary interacting regions on SE to the protein-protein interaction platform, including the N-terminal domain and the Mid domain (Fig. 1B,D).

The specific 6b/SE and 6b/AGO1 interactions probably suggest that not only RISC/AGO1 but also DCL1-SE-HYL1 are essential protein complexes that participate in virus-induced gene silencing (VIGS) to defend against offending viruses. Compared with other viral suppressors primarily targeting on AGO1, such as CMV2b and P0 (Zhang et al. 2006b; Baumberger et al. 2007; Bortolamiol et al. 2007), the 6b protein targets a wide range of host proteins residing inside the nucleus (Kitakura et al. 2002, 2008; Terakura et al. 2007). The ADP ribotransferase fold displayed by the 6b structure enables 6b to function as an

Figure 7. AK6b is a novel ADP-ribosylating toxin. (A) AK6b catalyzes ADP ribosylation of SE in the presence of GTP. The relative ADP ribosylation activity after each reaction is shown by the bars. The substrate, enzyme, cofactors, and ARF used for experiments are shown *below* the chart. (Lanes 1–4) Wild-type AK6b. (Lanes 5–9) Point mutations of AK6b. (B) In vitro pull-down assay of GST-AK6b interaction with His-AtARF. GST-AK6b was immobilized on GST beads and incubated with full-length His-ARF. Bound GST-AK6b was retrieved, and the association of His-ARF was monitored by Western blots using monoclonal antibodies against poly-His. (Lane 1) Input control. (Lane 2) Molecular marker size positions. (Lane 3) GST only. (Lane 4) GST-AK6b (protein batch 1). (Lane 5) GST-AK6b (protein batch 2). (C) Interaction of ARF and AK6b in *Arabidopsis* plants in vivo. Two-week-old seedlings expressing 35S::AK6b were treated overnight with 50 μM MG132. Total protein extracts were immunoprecipitated (IP) with polyclonal antibody to AK6b. Western blots were analyzed with a human ARF monoclonal antibody to detect *Arabidopsis* ARF in vivo. (Lane 1) Input, plant extract only. (Lane 2) Eluent pulled down from beads bound with AK6b antibody. (Lane 3) Eluent pulled down from beads without AK6b antibody bound (negative control). (D) Crystal structure of *Arabidopsis* ARF (AtARF). (Left panel) Cartoon representation of AtARF in complex with GDP-Mg²⁺. Switch1, switch2, and interswitch are colored in pink, blue, and red, respectively. (Middle panel) Ribbon presentation of superimposition of AtARF

in complex with GDP-Mg²⁺ with human ARF6 in complex with GDP-Mg²⁺. AtARF is colored in gray, whereas human ARF6 is colored in wheat. The color code for switch1, switch2, and interswitch is the same as that used in the *left* panel. (Right panel) Ribbon presentation of superimposition of AtARF in complex with GDP-Mg²⁺ with human ARF6 in complex with GTPγS. AtARF is colored in gray, whereas human ARF6 is colored in wheat. The color code for switch1, switch2, and interswitch is the same as that used in the *left* panel. (E) A hypothetical working model for ADP ribosylation of substrate catalyzed by 6b in the presence of ARF. (Left panel) The 6b protein trapped at cofactor-free conformation. (Right panel) The 6b protein trapped at the ARF-bound activation form. Highly conserved repeating Glu loop and target-binding loop used for substrates binding.

ADP-ribosylating toxin to target many host proteins with apparently completely different functions.

Interestingly, although both 6b and CMV2b target AGO1 to interfere with the miRNA pathway in plants, the primary target of 6b seems to be SE, because the miRNA expression levels in 35S::AK6b plants are dramatically decreased (Fig. 1B). This is not surprising, because SE functions upstream of AGO1.

6b belongs to a novel toxin family

Although having limited sequence homology, ADP-ribosylating toxins share common core structures for NAD⁺ binding (Fig. 3). However, the toxins can be divided into two groups, with the DT group represented by exotoxin A and the CT group represented by cholera toxin. The completely different sets of catalytic residues within the

active site used by these two different toxin groups (DT vs. CT) strongly suggest that the mechanisms of substrate recognition could be different (Deng and Barbieri 2008).

In the exotoxin A structure, the active site is composed of the characteristic residues Glu 553, His 440, Tyr 481, and Tyr 470 (Fig. 6B, middle panel). Glu 553 forms a hydrogen bond with the 2'OH of N-ribose of NAD⁺ and orientates the dinucleotide substrate for nucleophilic attack, whereas His 440, Tyr 481, and Tyr 470 stabilize the bound NAD⁺ substrate (Fig. 6B, middle panel). Moreover, a comparison of the structures of apo-exotoxin A and that of the EF2 complex demonstrated that the contact surface between exotoxin A and its EF2 target is rather small, and the conformational changes are marginal on NAD⁺ binding or EF2 binding or during the substrate catalysis process (Jørgensen et al. 2005, 2008a).

In contrast, in the cholera toxin structure, the active site is composed of the characteristic residues Arg 7, Ser 61, Glu 110, and Glu 112, which are occluded by the active site loop (residues 47–56, cholera toxin residue number) (Fig. 6B, right panel). Upon binding to its partner protein, such as ARF6-GTP, this loop swings out of the active site to expose residues for substrate binding and catalysis (Lobet et al. 1991; Cieplak et al. 1995; van den Akker et al. 1996; O'Neal et al. 2004, 2005).

The 6b protein contains a conserved central module resembling the overall structural similarity to the NAD⁺-binding pockets of all of the ADP-ribosylating toxins discovered so far, although there is no apparent sequence similarity between 6b and the ADP-ribosylating toxins. Moreover, 6b contains a completely different set of catalytic residues within the putative NAD⁺-binding pocket, suggesting that 6b may represent a novel family of ADP-ribosylating toxins (Fig. 6B, left panel). The well-conserved Glu (exotoxin A and cholera toxin) or His/Arg (exotoxin A/cholera toxin) is not found within the 6b active site pocket. Instead, highly conserved Tyr 66 and Tyr 153 residues are employed to supplant the functions of Glu and His/Arg residues, respectively. In addition, the critical highly conserved Thr 93 probably plays the same functional role as Tyr 470 at exotoxin A or Ser 61 at cholera toxin by forming hydrogen bonds with the catalytic Tyr 153 residue to facilitate ADP ribosylation (Fig. 6B, left panel). Our in vitro ADP ribosylation assay unambiguously demonstrated that the highly conserved Y66, T93, Y121, and Y153 play significant roles for ADP ribosylation in vitro (Fig. 7A, lane 5–8).

Furthermore, similar to cholera toxin, the active site pocket of 6b is occluded by the active site loop (residues 113–134) (Fig. 6A [left panel], B [left panel]). Therefore, significant conformational changes are required to expose this active site pocket to allow for NAD⁺ access. Consistent with this structural observation, 6b does not display any ADP ribosylation function in vitro on any target proteins discovered so far (Fig. 7A, lane 1; data not shown). Indeed, *Arabidopsis* ARF and GTP are both needed to trigger the conformational change of 6b for target binding (Fig. 7A, lane 4).

Potential roles of 6b on plant gene regulation and cell growth

6b proteins interact with many different host proteins involved in the expression of genes related to plant cell proliferation, and various hypotheses have been proposed to explain the effects of 6b on plant cell growth. The repeating Glu residue loop (residues 164–184) was reported to interact with NtSIP1, which contains the trihelix motif (Kitakura et al. 2002), and NtSIP2, which is homologous to the TNF1 protein (Kitakura et al. 2008). However, the 6bΔA mutant protein (lacking the repeating Glu residue loop 164–184) retains the ability to interact with *Arabidopsis* histone H3, SE, and AGO1 (Terakura et al. 2007; this study). It appears that there are two groups of proteins interacting with 6b: one group targeting this acidic loop, and the other group targeting other structural elements. The acidic loop (residues 164–184), which may serve as

a structural scaffold for protein–protein or protein–nucleic acid interaction, is disordered in both the AK6b and AB6b structures. In contrast, we identified one well-ordered loop (residues 40–55) that serves as a structural scaffold for *Arabidopsis* histone H3, AGO1, and SE binding (Fig. 4; Supplemental Fig. S2).

The fact that the 6b protein targets a wide range of diversified host proteins in plants suggests that the phenotypes generated by 6b expression are a consequence of its interaction with a number of proteins. On one hand, 6b might function as a transcriptional coactivator/mediator by binding to proteins involved in the transcription machinery, and this binding may be mediated by the repeating acidic Glu residue loop. On the other hand, 6b might function as an ADP-ribosylating toxin by targeting key components in the miRNA processing/silencing machinery using the well-ordered extended loop (residues 40–55) as an interacting platform. The ectopic expression of a number of genes—such as class I *KNOX*, *CUC*, and cell cycle-related genes—caused by 6b expression probably depends on the general ADP ribosylation activity of 6b. Because covalent modifications of host proteins catalyzed by ADP ribosylation often result in an inactivation of the targets, the ADP ribosylation activity of 6b is likely responsible for the various developmental abnormalities of 35S::AK6b plants, including leaf curling, serration, and dwarfism. ADP ribosylation modification of host target proteins by 6b may result in an inactivation of the target proteins that eventually changes cell physiology and even produces tumors (Fig. 7E). Consistent with this hypothesis, the mutations on the conserved ADP ribosylation catalytic residues Tyr 66 or Thr 93 lead to a loss of 6b oncogenicity (Helfer et al. 2002). Moreover, the abnormal growth of calli induced by 6b is probably also dependent on the ADP ribosylation activity, because the 6bΔC mutant protein (lacking residues 164–208), which disrupts the overall ADP ribosylation fold, did not elicit any abnormal growth of calli (Kitakura et al. 2002; Terakura et al. 2007). Alternatively, the loss of phenotype may be due to the instability of these 6b mutant proteins in vivo, although the mutant proteins expressed at the *E. coli* system show protein stabilities similar to those of the wild type.

Although the 6b gene might play only a minor role in crown gall formation, it could play a much more important role in tumorigenicity in certain host species (Garfinkel et al. 1981; Joos et al. 1983; Hooykaas et al. 1988; Tinland et al. 1989, 1992). These observations are also consistent with our structural and functional evidence that the 6b protein might be a novel ADP-ribosylating toxin that has certain host target specificity. A major challenge in the future will be to determine the crystal structure of the quadruple complex 6b–ARF–GTP–substrate, which will eventually help to uncover the molecular mechanism of 6b action.

Materials and methods

Plant material

Arabidopsis thaliana [*se-1*, *ago1-27* mutant, and all 6b transgenic plants] was grown under 16-h light and 8-h dark conditions at 22°C.

Construction of plasmids

Plasmids and their relevant characteristics are listed in Supplemental Table S1. All manipulations for plasmid construction were performed using standard techniques (Sambrook et al. 1989), and constructs were verified by sequencing. *E. coli* DH5 α was used as a host for plasmid construction.

A series of 6b-related vectors was constructed as follows. Full-length *AK6b* from pTiAKE10 and *AB6b* from pTiAB4 were cloned using the same specific primers: 5'-ATGACGGTTCC TACTTGG-3' and 5'-CTAACGTGTGAGATGGCAC-3'. Mutants of *AK6b* were prepared by overlapping PCR. The PCR fragments for protein expression in *E. coli* with an His tag, GST tag, or MBP tag were digested with NdeI/XhoI, EcoRI/XhoI, or EcoRI/HindIII. The resulting fragments were ligated into pET-28b (kanamycin selection; Novagen), pGEX-6p-1 (ampicillin selection; Novagen), or pMAL-c2X (ampicillin selection; Novagen) vectors, respectively, to yield pET-AK6b and pET-AB6b; pGEX-AK6b, pGEX-AB6b, and pGEX-200; and pMAL-40 and pMAL-164. The PCR fragments for the transformation of *Arabidopsis* were digested with XbaI/SacI. The resulting fragments were linked to the cauliflower mosaic virus P35S in the binary vector pBI121 to yield *p121-AK6b*, *p121-40*, *p121-66*, *p121-93*, *p121-116*, *p121-121*, *p121-123*, and *p121-146*.

ARF1A1C was cloned from *Col-0* cDNA using the primers 5'-GATGCACatATGGGGTTGTTCATTCGG-3' and 5'-GTAGCT ctcgagTTACTATGCCTTGCTTGCAT-3'. PCR product was digested with NdeI/XhoI and inserted into pET-28b to yield N-His-ARF.

Production of transgenic plants

Supplemental Table S1 lists the binary plasmids used in this study. Mutated versions of *AK6b* were generated by overlapping PCR. *A. thaliana* (*Col-0*) plants were transformed by *Agrobacterium* (EHA105 strain)-mediated infiltration using the floral dipping method (Clough and Bent 1998; Zhang et al. 2006a).

Generation and selection of antibodies

The SE core domain (195–544) and *Thermus* thermophilic AGO-like protein were subjected to monoclonal antibodies preparation, whereas full-length *AK6b* was subjected to polyclonal antibody preparation using standard protocols. We obtained eight different monoclonal antibodies against the *Thermus* thermophilic AGO-like protein and six different monoclonal antibodies against the SE core domain (195–544). We systematically screened all eight *Thermus* thermophilic AGO-like antibodies against the wild-type *Arabidopsis* and Flag-AGO1 line. At the end, we obtained one antibody that specifically recognizes AtAGO1 (Supplemental Fig. S1). Similarly, we were able to get a monoclonal antibody against full-length SE.

In vitro pull-down assays and co-IP experiments

Fifty micrograms of His-tagged target proteins was preabsorbed in the binding buffer, including amylose resin (Biolabs) or glutathione-agarose beads (GE Healthcare), before the MBP-tagged or GST-tagged bait proteins were added. Pulled-down proteins were resolved by SDS-PAGE and detected by Western blot.

For the co-IP experiments, ~0.3 g of frozen tissue was homogenized in 0.75 mL of lysis buffer and spun down to remove debris. The supernatant was precleared with 100 μ L of protein-A gel for 1 h, and then collected to be incubated with 4 μ g of anti-AK6b antibody for 1 h. The antibody associated with the protein complex was pulled down with 40 μ L of protein-A gel, followed

by washing five times in the lysis buffer. The protein complex was eluted for 5 min on a 100°C heat block. The eluted protein mixture was resolved on 15% SDS-PAGE gel and transferred onto a PVDF membrane. Western blots were analyzed with a polyclonal antibody specific to SE or H3, or with a monoclonal antibody specific to ARF.

Northern blot analysis

For analysis of miRNAs, total RNA was separated on denaturing 15% TBE-urea gels. Probe detection was performed using the LightShift Chemiluminescent EMSA kit (ThermoFisher Scientific, Inc.) according to the manufacturer's protocol.

Real-time RT-PCR

Total RNA was isolated from the aerial parts of 20-d-old wild-type, *se-1*, and *35S::AK6b* seedlings using Trizol reagent (Invitrogen). Reverse transcription was performed with 2 μ g of total RNA using the First Strand cDNA Synthesis kit (Fermentas, Inc.). PCR was carried out in the presence of the dsDNA-specific dye SYBR Green (Applied Biosystems). Amplification was monitored in real time with the 7500 Real-Time PCR System (Applied Biosystems).

Protein purification

His or His-SUMO fusion proteins include His-AK6b, His-AB6b, His-SE, and His-ARF. GST-tagged proteins include GST-AK6b, GST-AB6b, GST-AK6b (Δ 200–208), GST-AK6b (Y66A), GST-AK6b (T93A), GST-AK6b (Y121A), GST-AK6b (D146A), GST-AK6b (Y153A), GST-SE-Mid, and GST-SE-NT. MBP fusion proteins include MBP, MBP-AK6b (Δ 40–55), MBP-AK6b (Δ 164–184), MBP-AGO1-PolyQ, MBP-AGO1-NT, MBP-AGO1-PAZ, MBP-AGO1-Mid, MBP-AGO1-PIWI1, and MBP-AGO1-PIWI2. All proteins were expressed in *E. coli* and purified using affinity columns, followed by size exclusion column chromatography.

In vitro ADP ribosylation assay

The in vitro ADP ribosylation assay was carried out as described previously (Armstrong and Merrill 2001). Each reaction contained 350 μ M ϵ -NAD⁺, 20 μ M SE, 1 μ M ARF1A1C, and 500 μ M GTP or GDP. Following temperature equilibration for 10 min in buffer (20 mM Tris-HCl at pH 7.8) the reaction was initiated by the addition of wild-type AK6b or its mutant derivatives (1 μ M). Fluorescence was measured using an xMarkTM Microplate Spectrophotometer (Bio-Rad) with a 305-nm excitation wavelength.

Crystallization and data collection

Crystals of AK6b were grown by hanging-drop vapor diffusion at 20°C. Typically, a 2- μ L hanging drop contained 1.0 μ L of protein (8 mg/mL) mixed with 1.0 μ L of reservoir containing 0.3 M magnesium formate and 0.1 M Bis-tris buffer (pH 5.9), and was equilibrated over 1 mL of reservoir solution. The crystals grew to a maximum size of 0.2 mm $\times$ 0.2 mm $\times$ 0.1 mm over the course of 2 d.

Crystals of AB6b were grown by hanging-drop vapor diffusion at 20°C. Typically, a 2- μ L hanging drop contained 1.0 μ L of protein (12 mg/mL) mixed with 1.0 μ L of reservoir containing 0.35 M ammonium dehydrate phosphate and 0.1 M sodium citrate (pH 5.8), and was equilibrated over 1 mL of reservoir solution. These crystals grew to a maximum size of 0.05 mm $\times$ 0.05 mm $\times$ 0.02 mm over the course of 7 d.

Crystals of *Arabidopsis* ARF were grown with GDP by hanging-drop vapor diffusion at 20°C. Typically, a 2- μ L hanging drop contained 1.0 μ L of protein (15 mg/mL) mixed with 1.0 μ L of reservoir containing 25% PEG6000, 0.2 M magnesium chloride, and 0.1 M HEPES (pH 7.2), and was equilibrated over 1 mL of reservoir solution. These crystals grew to a maximum size of 0.15 mm $\times$ 0.15 mm $\times$ 0.02 mm over the course of 10 d.

For data collection, AK6b crystals were flash-frozen (100°K) in the above reservoir solution supplemented with 30% glycerol. A total of 360 frames of 1° oscillation was collected for each crystal per wavelength. A total of three wavelengths' data was collected, corresponding to selenium peak (0.9790 Å), inflection (0.9794 Å), and remote (0.96 Å). The data were collected on the beamline X12C at the National Synchrotron Light Source at Brookhaven National Laboratory, and was processed by HKL2000 (<http://www.hkl-xray.com>). The crystals belong to space group P3₂21, with the unit cell parameters listed in Table 1.

AB6b crystals were flash-frozen (100°K) in the reservoir solution supplemented with 30% glycerol. A total of 360 frames of 1° oscillation was collected at wavelength 1.1 Å on the beamline X29 at the National Synchrotron Light Source at Brookhaven National Laboratory, and was processed by HKL2000 (<http://www.hkl-xray.com>). The crystals belong to space group P2₁2₁2, with the unit cell parameters listed in Table 1.

Arabidopsis ARF crystals were flash-frozen (100°K) in the reservoir solution. A total of 360 frames of 1° oscillation was collected at wavelength 1.075 Å on the beamline X29 at the National Synchrotron Light Source at Brookhaven National Laboratory, and was processed by HKL2000 (<http://www.hkl-xray.com>). The crystals belong to space group P2₁2₁2, with the unit cell parameters listed in Table 1.

Structure determination

The crystal structure of AK6b was determined by MAD using SOLVE/RESOLVE (<http://www.solve.lanl.gov>), and the MAD phase was calculated and improved by density modification, assuming a solvent content of ~48% using the SHARP program (<http://www.globalphasing.com>). The crystal structure of AB6b was determined by molecular replacement, with the AK6b structure as the search model. The crystal structure of *Arabidopsis* ARF was determined by molecular replacement, with the human ARF6 structure (PDB ID: 1RRF) as the search model. The models were built by using the program O and were refined using REFMAC/CCP4. The R-free set contained 5% of the reflections, chosen at random. The model comprises residues 5–208 (AK6b), 1–204 (AB6b), or 4–180 (AtARF). The disordered region—including loop segment 166–182 (AK6b), 167–179 (AB6b), or 70–76 (AtARF)—was not included in the model.

Further information about the mutant lines, sample preparation, primer sequences, reactions, and data analysis can be found in the Supplemental Material.

Accession codes

Coordinates and the structure factors have been deposited to PDB with accession codes 3AQ2 (AB6b), 3AQ3 (AK6b), and 3AQ4 (*Arabidopsis* ARF1A1C).

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