Aurora kinases play a key role in chromosome segregation and cytokinesis. In plants, three Aurora kinases (AtAUR1–AtAUR3) have been identified in Arabidopsis thaliana. Here, we report an AtAUR2 splicing variant (AtAUR2S), which lacks the fourth exon encoding a part of the kinase domain of AtAUR2. AtAUR2S was shown to have lost its kinase activity to phosphorylate histone H3 at Ser10; however, it maintained its ability to bind to histone H3. The localization pattern of AtAUR2S was the same as that of AtAUR2. The findings suggest that AtAUR2S affects cell division by competing with AtAUR2.

Keywords: Alternative splicing — Arabidopsis thaliana — Aurora kinase — Histone H3.

Abbreviations: BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; GST, glutathione-S-transferase; PBS, phosphate-buffered saline; RT–PCR, reverse transcription–PCR.

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Aurora kinase is a serine/threonine kinase that plays essential roles in cell division (Carmena and Earnshaw 2003). In vertebrates, Aurora kinases are classified into three types, Aurora A, B and C. Strict regulation of Aurora kinases is necessary for proper cell division, and not only depletion mutants but also overexpression of Aurora kinases result in seriously deleterious phenotypes, e.g. formation of multinuclear cells, aneuploidy and misorientation of spindles, which are the main causes of cancer (Carmena and Earnshaw 2003). The transcriptional and expression levels of mRNA and protein of Aurora kinases are regulated during the cell cycle (Bischoff et al. 1998, Tanaka et al. 2002, Kimura et al. 2004). Aurora A is transcriptionally up-regulated during mitosis (Tanaka et al. 2002) and degraded by APC/C^Cdh1 regulation after metaphase (Honda et al. 2000, Castro et al. 2002, Littlepage and Ruderman 2002, Taguchi et al. 2002). Aurora kinase activities are regulated by phosphorylation (Walter et al. 2000, Yasui et al. 2004) and dephosphorylation (Katayama et al. 2001, Sugiyama et al. 2002), and are associated with activators (Eyers et al. 2003, Hirota et al. 2003, Honda et al. 2003, Pugacheva and Golemis 2005) and negative regulators (Chen et al. 2002, Sugiyama et al. 2002, Eyers et al. 2003).

In Arabidopsis thaliana, three Aurora kinase genes (AtAUR1, 2 and 3) have been identified (Demidov et al. 2005, Kawabe et al. 2005). Similarly to animal Aurora kinases, the kinase domains are highly conserved but the N-terminal regions are variable. AtAUR1 and AtAUR2 show a large degree of similarity in the amino acid sequences of their kinase domains (95% sequence identity). All three AtAURs have kinase activity to phosphorylate histone H3 in vitro (Kawabe et al. 2005). Both transcripts and proteins are abundant in young roots, flower buds and flowers, indicating that they are strongly expressed in dividing cells (Demidov et al. 2005). Analyses of localization patterns using green fluorescent protein (GFP)-fused proteins in tobacco BY-2 cultured cells showed that AtAUR1 and AtAUR2 are localized at the nuclear membrane during interphase and move to spindle microtubules during mitosis. Their subcellular localization is similar, except that only AtAUR1 localizes in the young cell plate (Demidov et al. 2005, Kawabe et al. 2005). The localization patterns of AtAUR1 and AtAUR2 are similar to that of Aurora A (Carmena and Earnshaw 2003). Here, we show that an alternative splicing variant, AtAUR2S, is expressed at a low level with the same localization pattern as that of AtAUR2 and can bind to a substrate, histone H3, although it loses its kinase activity.

When PCR with the primers AtAUR2-F1 and -R1 was performed using a phage clone of the Arabidopsis cDNA library constructed from the hypocotyl of a 3-day-old
seedling (Kawabe et al. 2005), two different amplified fragments were detected. The larger fragment corresponded to the previously reported \textit{AtAUR2} and the smaller fragment was designated as \textit{AtAUR2S}. The sequence of \textit{AtAUR2S} is identical to that of \textit{AtAUR2} but lacks a 96 bp region from nucleotide position 364–459 (Fig. 1a). This 96 bp region corresponds to the entire length of exon 4 in the genomic sequence, indicating that the short transcript was caused by alternative splicing. No such deletion transcripts were detected with \textit{AtAUR1} by similar PCR analysis. Although the exons of \textit{AtAUR1} and \textit{AtAUR2} show a high level of identity, the introns show almost no sequence similarities (Fig. 1b). Thus, the \textit{AtAUR2}-specific alternative splicing might have been caused by mutations

Fig. 1 Structure of two transcript forms of the \textit{AtAUR2} gene in \textit{A. thaliana}. (a) Structure of genomic and transcript \textit{AtAUR2}. The schematic gene structure of \textit{AtAUR2} is shown at the top. The genomic exon–intron structure and spliced forms of \textit{AtAUR2} and \textit{AtAUR2S} are presented. The amino acid sequences of \textit{AtAUR1} (accession No. BAE00019), \textit{AtAUR2} (accession No. BAE00020), \textit{AtAUR2S} and human Aurora A (accession No. O14965; \textit{H. Aurora A}) around the deleted region of \textit{AtAUR2S} are shown below the gene structure. Human Aurora A secondary structure elements are labeled below the alignment, and the activation motif region and protein kinase A phosphorylation site at threonine are shown in bold (Walter et al. 2000). (b) Dot matrix comparison of the \textit{AtAUR1} and \textit{AtAUR2} genomic sequences. Dots were plotted when more than seven bases were identical in 10 bp. Gene structures are shown beside the matrix where exons are shown in boxes. The alternatively spliced exon (4 for \textit{AtAUR2}) is shown by dotted lines.
in intron sequences accumulated after duplication of AtAUR1 and AtAUR2. Compared with recognition sites of a spliceosome complex in AtAUR1 and AtAUR2, the sequence in the branch site was changed from GCTAT (position −30 to −26 bp upstream from the 3′ splice site of intron 4 in AtAUR1) to CTGAT (position −26 to −23 bp upstream from the 3′ splice site of intron 3 in AtAUR2). Considering that the frequency of GTCAT in the branch site of A. thaliana is higher than that of CTGAT (Lim and Burge 2001), it is possible to weaken the recognition by a spliceosome complex in intron 3 of AtAUR2. Considering that the amino acid sequence of this deleted exon is highly conserved among Aurora kinase genes of plants and animals, this region is thought to play an important role.

We therefore investigated the kinase activity of AtAUR2S in vitro by detecting a phosphorylated Ser10 of the glutathione-S-transferase (GST)–H3 tail (H3S10ph). H3S10ph was detected only when GST–AtAUR2 was added to the GST–H3 tail. With GST–AtAUR2S, H3S10ph was below the detection level (Fig. 2a), indicating that the deletion of this region of AtAUR2 results in a lack of kinase activity. Crystal structures of the human Aurora A kinase domain show a typical kinase fold comprised of an N-terminal β-strand domain and a C-terminal α-helical domain, linked together by a hinge region that is the catalytic active site (Cheetham et al. 2002). The deleted exon 4 is the region that connects the hinge region and the activation loop (Fig. 1a), and therefore the deletion in AtAUR2S may lead to conformational changes and subsequently to loss of kinase activity.

It is quite probable that AtAUR2S interacts with its substrates, although it cannot phosphorylate them because the phosphorylation and substrate-binding sites are not identical (Kunitoku et al. 2003). To confirm the binding ability of AtAUR2S, far-Western analysis was performed using purified GST-fused AtAUR proteins and total histone H3 (Fig. 2b). Because all three AtAUR proteins can phosphorylate histone H3 (Kawabe et al. 2005), they should bind histone H3 in vitro. With anti-GST antibodies, signals were detected in both the AtAUR2 and AtAUR2S assays, indicating that the deletion of exon 4 does not influence the ability of AtAUR2 to bind to total histone H3 (Fig. 2b). These in vitro analyses reveal that the deletion in the splicing variant leads to a lack of kinase activity but the protein maintains its ability to bind to its substrates.

Because the subcellular localization of Aurora A is determined mainly by the N-terminal domain (Giet and Prigent 2001), AtAUR2S could have the same localization patterns as AtAUR2. To confirm this, GFP fused with AtAUR2S under a cauliflower mosaic virus (CaMV) 3S promoter was transformed into cultured tobacco BY-2 cells. Stable lines expressing GFP–AtAUR2 and GFP–AtAUR2S were observed using a fluorescence microscope (Fig. 3). Mitotic phases were determined by 4′,6-diamidino-2-phenylindole (DAPI) staining. During interphase, AtAUR2S was located in the nucleus and the nuclear periphery region. At metaphase, GFP fluorescence was detected in the mitotic spindle and, at anaphase, in the spindle halves. These subcellular localization patterns were completely consistent with those of AtAUR2, suggesting normal localization even after deletion of exon 4. AtAUR2S could also associate with the mitotic spindles during mitosis.

Although we observed cell growth, cell division and chromosome dynamics, the BY-2 cells overexpressing AtAUR2S showed no obvious phenotype. However, non-functional proteins with binding ability only could disturb binding of the native protein, and thus the functions of proper cell division. To examine whether the expression pattern of the splicing variant differs in different organs, reverse transcription–PCR (RT–PCR) with the primers AtAUR2-F2 and -R2 was performed (Fig. 3c). Two amplified fragments (Fig. 3c, upper band, AtAUR2; lower band, AtAUR2S) were observed in all organs; however, the lower bands were very faint, suggesting that accumulation of the AtAUR2S transcript in each organ is much less than that of AtAUR2. Although the expression level of AtAUR2S was low, AtAUR2S showed relatively high expression in leaves and stems. Northern hybridization
analysis revealed that mRNA of \textit{AtAUR2} was strongly expressed in developing organs including the roots, flower buds and flowers (Demidov et al. 2005). In contrast, mRNA of \textit{AtAUR2S} was expressed at a relatively high level in already matured organs with low division activity. This difference suggests strict regulation of splicing forms in different plant tissues. Moreover, the low expression in actively developing organs suggests that \textit{AtAUR2S} can alter strictly regulated cell divisions and cause deleterious effects. The normal phenotype of transformed BY-2 cells could be related to the nature of this cultured cell strain, which shows high and fast cell division activity (Nagata et al. 1992). In addition, the effect of Aurora kinase inhibitor on the phenotype of BY-2 cells is very weak, suggesting that a small amount of active kinase could retain proper regulation of cell division and other down-regulated events in this cell line (Kurihara et al. 2006).

Alternative splicing was also reported in human Aurora C, producing the splicing variant Aurora C-SV, which is missing part of the first exon. Aurora C-SV showed the same subcellular localization as that of Aurora C but its abundance was very low and its kinase activity was significantly decreased (Yan et al. 2005). The reports of an alternative variant without kinase activity in humans and \textit{A. thaliana} suggest that these variants function in the regulation of plant and animal Aurora kinases, although the function of each variant remains unknown. One possible explanation is that this splice form acts as a substrate-competitive inhibitor of phosphorylation. Comparisons of the amino acid sequence of \textit{AtAUR2S} with that of Aurora A revealed that \textit{AtAUR2S} has a substrate-binding site (N-terminal region) and an auto-phosphorylation site (Fig. 4). Because the distance between both amino acid ends of the deleted region is approximately 10 Å, it is considered that the conformation of \textit{AtAUR2S} is not entirely changed compared with \textit{AtAUR2} (Fig. 4, red dotted line). However, \textit{AtAUR2S} lacked the region between the ATP-binding site and activation loop (Fig. 4, red and magenta). Therefore, it is suggested that the phosphate group of ATP could not be transferred to the Ser/Thr residue of the substrate. Considering that \textit{AtAUR2S} was also shown to lack four out of 24 ATP-binding residues and one out of two Mg\textsuperscript{2+} ion-binding residues (Fig. 4, magenta), it is possible that \textit{AtAUR2S} does not bind to ATP (Fig. 4, arrows). The kinase-inactive mutants of Aurora kinases have been shown to function as dominant-negative inhibitors (Meraldi et al. 2002, Murata-Hori and Wang 2002). Thus, \textit{AtAUR2S} could be a strong competitor of \textit{AtAUR2}, being under strict spatio-temporal regulations that resist \textit{AtAUR2} functions.

Alternative splicing is an important mechanism that can contribute to the proteome diversity and regulate gene expression post-transcriptionally. It has been estimated that approximately 40% of human genes are alternatively spliced. In contrast, far fewer alternatively spliced genes have been reported in plants. However, recent studies showed that several alternatively spliced genes in \textit{Arabidopsis} encode proteins with regulatory functions and many stress-related genes are alternatively spliced (Kazan 2003). Alternative splicing could have significant roles in tissue specificity and cell cycle progression in plants as in mammals. For instance, tobacco cyclin-dependent kinase (CDK) inhibitor NtKIS1a and its alternative spliced variant NtKIS1b display different expression patterns during

![Fig. 3 Expression and localization patterns of AtAUR2 and AtAUR2S. (a, b) Subcellular localization of AtAUR2 (a) and AtAUR2S (b) in tobacco BY-2 cells, at inter phase, metaphase and anaphase. The top column shows DNA staining with DAPI and the middle column shows GFP fluorescence. The bottom column shows merged images. Merged images of DNA (blue) and GFP (green) are shown in color. Scale bars: 10 μm. (c) Expression patterns of AtAUR2 and AtAUR2S in Arabidopsis thaliana. Total RNA was extracted from a 3-day-old seedling, the roots, a leaf, stem, flower bud and flower. Cloned AtAUR2 and AtAUR2S cDNAs were used as a control. Expression was monitored by RT–PCR. The upper and lower arrows indicate the band positions of \textit{AtAUR2} and \textit{AtAUR2S} at the right of the gel image, respectively.](image-url)
the cell cycle. NtKIS1b is a strong competitor of NtKIS1a regarding the inhibition of CDK activity. This suggests that NtKIS1a and NtKIS1b regulate cell cycle progression differently (Jasinski et al. 2002). Further studies, especially of detailed localization patterns and mechanisms that inhibit the splice variant in actively dividing tissues, will reveal details of AtAUR2S function and the regulation of mitosis by plant Aurora kinases.

Materials and Methods

Cloning of AtAUR2 cDNA was conducted as previously described by Kawabe et al. (2005). The same primer combination as for purification of AtAUR2 (F1, 5′-ATCTGTCATCAAGGGC GGCTCAGA-3′; R1, 5′-AATACCTTTGGAAACACAGCA-3′) was used. Using this primer combination, two bands were amplified from the AtAUR2 and AtAUR2S transcripts, respectively. The lower band was cloned into pDONR 221 using the adaptor PCR method of gateway technology (Invitrogen) according to the manufacturer’s recommendations.

Cloning of the expression vectors for AtAUR2 and A. thaliana histone H3 was conducted as previously described, except for cloning of the AtAUR2 and AtAUR2S genes into the pDEST15 expression vector (Kawabe et al. 2005, Kurihara et al. 2006). The GST–H3 tail construct was obtained by cloning a PCR fragment corresponding to amino acids 1–48 of A. thaliana histone H3 into the pDONR 221 vector, and then into the pDEST15 expression vector. Purification of recombinant proteins (GST–AtAUR2, GST–AtAUR2S, His-tagged total histone H3 and GST–H3 tail) was conducted using the same procedures previously described (Kurihara et al. 2006) except for expression of GST–AtAUR2 and GST–AtAUR2S in Escherichia coli C43 (DE3) (Miroux and Walker 1996).

The in vitro kinase assay was performed using purified recombinant GST–AtAUR2 and GST–AtAUR2S, GST–H3 tail as the substrate, and 0.2 mM ATP in XBE2 buffer (10 mM K+ HEPES, 50 mM sucrose, 100 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, 2 mM K+ EGTA, pH 7.7) at 25°C for 30 min. Phosphorylated histone H3 was determined by immunoblotting using rabbit anti-H3S10ph antibodies (Upstate Biotechnology) and AP-labeled goat anti-rabbit IgG antibodies (Vector).

The in vitro binding assay was performed by far-Western blotting based on the procedures of Muchardt et al. (2002). The purified total histone H3 was resolved by SDS–PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4°C in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 1% bovine serum albumin (BSA), then each lane was dissected and incubated overnight at 4°C with 0.6 μg ml⁻¹ recombinant GST–AtAUR proteins or GST protein alone in PBS containing 1% Tween-20 and 5% BSA. Retained GST–AtAUR proteins were detected using goat anti-GST antibodies (Amersham Biosciences) and AP-labeled rabbit anti-goat IgG antibodies (Vector).

Tobacco BY-2 cells (Nicotiana tabacum cv. Bright Yellow-2) were maintained as previously described by Nagata et al. (1992) and cultured in modified Linsmaier and Skoog medium in a rotary shaker at 25°C in the dark. Vectors expressing C-terminal GFP fusion proteins were constructed with a destination vector, which is a binary vector designed for making GFP fusion proteins (pGWB5; Kawabe et al. 2005). Transformation of BY2 cells and observation of GFP-fused proteins were conducted as previously described by Kawabe et al. (2005).

Isolation of total RNAs and cDNA synthesis were conducted as previously described by Fujimoto et al. (2005). Total RNAs from six stages of A. thaliana were purified and then used to synthesize cDNAs. RT–PCR of the cDNA of six different development stages was performed using AtAUR2-specific primers (F2, 5′-TGATTTTTGGGTGGTTATCTCAGG TGGAAAGGTAAATC-3′; R2, 5′-ATCTCCAGG TGGAAAGGTAAATC-3′). PCR conditions were as follows: one cycle of 94°C for 1 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and one cycle of 72°C for 1 min.

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