Berberine enhances defects in the establishment of leaf polarity in asymmetric leaves1 and asymmetric leaves2 of Arabidopsis thaliana

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Abstract Leaves develop as flat lateral organs from the indeterminate shoot apical meristem. The establishment of polarity along three-dimensional axes, proximal–distal, medial–lateral, and adaxial–abaxial axes, is crucial for the growth of normal leaves. The mutations of ASYMMETRIC LEAVES1 (AS1) and AS2 of Arabidopsis thaliana cause defects in repression of the indeterminate state and the establishment of axis formation in leaves. Although many mutations have been identified that enhance the adaxial–abaxial polarity defects of as1 and as2 mutants, the roles of the causative genes in leaf development are still unknown. In this study, we found that wild-type plants treated with berberine produced pointed leaves, which are often observed in the single mutants that enhance phenotypes of as1 and as2 mutants. The berberine-treated as1 and as2 mutants formed abaxialized filamentous leaves. Berberine, an isoquinoline alkaloid compound naturally produced in various plant sources, has a growth inhibitory effect on plants that do not produce berberine. We further showed that transcript levels of meristem-specific class 1 KNOX homeobox genes and abaxial determinant genes were increased in berberine-treated as1 and as2. Berberine treated plants carrying double mutations of AS2 and the large subunit ribosomal protein gene RPL5B showed more severe defects in polarity than did the as2 single mutant plants. We suggest that berberine inhibits (a) factor(s) that might be required for leaf adaxial cell differentiation through a pathway independent of AS1 and AS2. Multiple pathways might play important roles in the formation of flat symmetric leaves.

Keywords Adaxial–abaxial polarity · asymmetric leaves2 · Berberine · Coptisine · Leaf development · Quadruplex DNA

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Introduction

Leaves develop as flat lateral organs from the shoot apical meristem, and the establishment of polarity along three-dimensional axes, proximal–distal, medial–lateral and adaxial–abaxial, is crucial for the growth of normal leaves (Steeves and Sussex 1989; Hudson 2000; Tsukaya 2006; Bowman and Floyd 2008; Szakonyi et al. 2010). Thus plants appear to exploit common mechanisms that are responsible for the establishment of these axes during leaf development. Genes that are involved in the formation of each of the three axes have been identified and their functions have been analyzed (Waites et al. 1998; Bowman and Smyth 1999; Sawa et al. 1999; Byrne et al. 2000; Nemhauser et al. 2000; Eshed et al. 2001; Kerstetter et al. 2001; McConnell et al. 2001; Ikawa et al. 2002; Pecker et al. 2005; Garcia et al. 2006). The asymmetric leaves1 (asl) and as2 mutants of Arabidopsis thaliana (A. thaliana) produce leaves abnormally in axis formation, and these mutants have pleiotropic phenotypes (Rédei and Hirono 1964; Tsukaya and Uchimiya 1997; Serrano-Cartagena et al. 1999; Byrne et al. 2000, 2001, 2002; Ori et al. 2000; Semiarti et al. 2001; Iwakawa et al. 2002, 2007; Matsumura et al. 2009; Ikezaki et al. 2010). Analyses by DNA microarray and real-time quantitative RT-PCR (qRT-PCR) revealed that a mutation of either AS1 or AS2 results in an increase in the transcription levels of genes for the abaxial determinants, ETTIN/AUXIN RESPONSE FACTOR3 (ETT/ ARF3), KANADI2 (KAN2) and YABBY5 (YAB5) as well as the class 1 KNOX genes, BREVIPEDICELLUS (BP), KNAT2, and KNAT6 (Iwakawa et al. 2007; Takahashi et al. 2008). The class 1 KNOX genes (BP, KNAT2, KNAT6, and SHOOT MERISTEMLESS [STM]) are expressed in the shoot apical meristem and thought to function in maintaining the indeterminate cell state (Lincoln et al. 1994; Long et al. 1996; Byrne et al. 2000; Ori et al. 2000; Semiarti et al. 2001; Ikezaki et al. 2010). AS1 encodes a myb domain protein and AS2 encodes a plant-specific protein with an AS2/LOB domain, both of which are nuclear proteins (Byrne et al. 2000; Iwakawa et al. 2002). These proteins form a complex structure (Yang et al. 2008) (hereinafter referred to as AS1–AS2) that is involved in the establishment of proper axis formation. In addition, some mutations of genes alter leaf patterning and axis formation in the absence of either AS1 or AS2: namely, ribosomal protein genes, genes involved in chromatin remodeling, histone modification, and small RNA biogenesis, and others (Ori et al. 2000; Xu et al. 2003, 2006; Kidner and Martienssen 2005; Li et al. 2005; Garcia et al. 2006; Huang et al. 2006; Yang et al. 2006; Ueno et al. 2007; Pinon et al. 2008; Yao et al. 2008; Pérez-Pérez et al. 2010; Horiguchi et al. 2011a, b; Kojima et al. 2011; Szakonyi and Byrne 2011; Ishibashi et al. 2012). Although the responsible wild-type genes also have important roles in the maintenance of the plants themselves, the roles of these wild-type genes in the establishment of leaf polarity are still unclear.

Alkaloids are nitrogen-containing low molecular weight substances that form a major class of plant-produced secondary metabolites. We screened for natural compounds that affect leaf development in A. thaliana and found that berberine, a benzylisoquinoline alkaloid, caused a pointed-leaf phenotype in wild-type plants. Berberine is naturally produced in various plant sources, such as the wood of Berberis, inner bark of Phellodendron, and rhizomes of Coptis (Grycová et al. 2007; Shitan and Yazaki 2007). The inhibitory effect of berberine on growth was reported in plant species that cannot produce berberine (Sakai et al. 2002). It has been reported that Arabidopsis plants might not produce berberine (Facchini et al. 2004); nevertheless, effects of berberine on A. thaliana have not been reported. For a half century, it has been known that berberine interacts with DNA and RNA (Klimek and Hnilica 1959; Yamagishi 1962; Kley and Hahn 1969; Wolfe et al. 1972). Berberine is used in biochemical research as a fluorescent probe of DNA and RNA (Hirakawa et al. 2005; Islam and Kumar 2008; Islam et al. 2009). Recently, it has been reported that berberine preferentially binds to triplex or quadruplex DNA (Lee et al. 1993; Ren and Chaires 1999; Franceschin et al. 2006; Arora et al. 2008; Ma et al. 2008; Bhadra and Kumar 2011). Guanine-rich sequences that presumably form quadruplex DNA are found throughout the genomes studied, including that of A. thaliana (Huppert and Balasubramanian 2005; Todd et al. 2005; Zhao et al. 2007; Mullen et al. 2010; Takahashi et al. 2012). It has been reported that sequences containing quadruplex DNA affect the transcript levels of genes located nearby (Siddiqui-Jain et al. 2002). In recent decades, berberine has been reported to possess antibacterial (Chae et al. 1999; Hu et al. 2000; Yan et al. 2008), anti-cancer (Anis et al. 2001), and antiphototoxidative activities (Kim et al. 2000). Berberine also induces apoptosis in several types of cells (Kuo et al. 1995; Hwang et al. 2006; Mantena et al. 2006) and has been used in medicines for both traditional and clinical applications. In spite of these reports and the broad uses of berberine, there have been no reports on the cause of its effects on growth inhibition and leaf morphology in plants that do not produce berberine.

In the present study, we report that wild-type Arabidopsis plants treated with berberine showed growth inhibition and changes of leaf morphology. Furthermore, we detected severe defects of leaf phenotypes related to the adaxial–abaxial polarity in asl and as2 mutants treated with berberine. We discuss these effects of berberine that interfered with normal leaf development in A. thaliana.
Results

Phenotypes of wild-type plants treated with berberine

When we treated plants with berberine (Fig. 1Aa), they displayed obviously severe effects of growth inhibition with decreased leaf size (Fig. 1Ab), similar to those observed in plants treated with trichostatin A (Ueno et al. 2007). As shown in Fig. 1B, the sizes of the first and second rosette leaves in plants at 18 days after sowing with 20 µM berberine were comparable to those at 12 days after sowing without berberine. Furthermore, the venation pattern of berberine-treated leaves was simpler than that of untreated leaves (Fig. 1B). We also observed pointed leaves at 20 and 25 µM concentrations of berberine similar to those observed in oligocella5-1 (oli5-1) and oligocella7-1 (oli7-1) mutant plants, each of which has a mutation in the large subunit of ribosomal protein gene RPL5A and RPL5B, respectively (Horiguchi et al. 2006; Fujikura et al. 2009; Fig. 1Cb, D; Table 1). These oli5-1 and oli7-1 mutants were identified as showing decreased palisade leaf cell numbers and pointed-leaf phenotypes. The leaf polarity defects of oli5-1 were rather more severe than those of oli7-1 (Horiguchi et al. 2011a). We quantified the pointed-leaf phenotype by measuring the tip angles of the first and second leaves. For wild-type plants grown without berberine, the mean tip angle was 123.7°, whereas those for berberine-treated wild-type and the oli5-1 and oli7-1 mutant plants were 118.7°, 98.6°, and 104.1°, respectively (p < 0.01). In this paper, we categorized leaves with tip angles less than 110° as ‘pointed leaves’. As shown in Fig. 1Db, there were no leaves with tip angles less than 110° seen in wild-type plants without berberine; whereas, 14% of wild-type leaves treated with berberine...
Table 1  Frequencies of plants with abnormal leaves and lethal phenotypes after growth on medium that contained berberine

| Berberine (µM) | Number of plants with pointed leaves | Number of plants with trumpet-like and filamentous leaves | Number of plants without true leaves |
|---------------|-------------------------------------|-------------------------------------------------------|-------------------------------------|
|               | 0        | 12.5    | 20      | 25    | 0        | 12.5    | 20      | 25    | 0        | 12.5    | 20      | 25    |
| Col-0 (WT)    | 0 ± 0    | 7 ± 9   | 8 ± 8   | 7 ± 7 | 0 ± 0    | 2 ± 1   | 3 ± 2   | 5 ± 6 | 0 ± 0    | 3 ± 2   | 11 ± 6 | 17 ± 14 |
| as1-1         | 0 ± 0    | 8 ± 6   | 8 ± 5   | 9 ± 6 | 0 ± 0    | 3 ± 3   | 13 ± 4  | 18 ± 10| 0 ± 0    | 1 ± 1   | 20 ± 21 | 27 ± 23 |
| as2-1         | 0 ± 0    | 5 ± 1   | 6 ± 2   | 3 ± 3 | 0 ± 0    | 8 ± 6   | 35 ± 16 | 28 ± 16| 0 ± 0    | 2 ± 3   | 14 ± 12 | 34 ± 21 |

Plants (n = 96) were used for each berberine treatment. Each value indicates the mean and the standard deviation of 4 batches of experiments.

(6 out of 42 leaves), 92% of oli5-1 leaves (34 out of 37 leaves), and 69% oli7-1 leaves (34 out of 42 leaves) had leaf-tip angles smaller than 110°.

Phenotypes of as1-1 and as2-1 plants treated with berberine

Mutations that cause pointed leaves, such as oli5-1 and oli7-1, have been previously reported to enhance the phenotypes of as1 and as2 mutants (Fig. 1Da; Pinon et al. 2008; Yao et al. 2008; Horiguchi et al. 2011a; Szakonyi and Byrne 2011; Ishibashi et al. 2012). Therefore, we examined the phenotypes of wild-type and as1-1 and as2-1 mutant plants treated without and with 12.5, 20, and 25 µM berberine. The results are given in Table 1. The pointed-leaf phenotype was found in 7, 8, and 7% of wild-type plants as well as in as1-1 and as2-1 plants treated with 12.5, 20, and 25 µM berberine, respectively (Table 1). Wild-type plants seldom showed radialized leaves (Table 1), whereas 3, 13, and 18% of as1-1 and 8, 35, and 28% of as2-1 plants had trumpet-like leaves and filamentous leaves indicative of a defect in adaxial–abaxial polarity at 12.5, 20, and 25 µM berberine, respectively (Fig. 2Ac, Ad, Ae, Af; Table 1). We also observed plants without true leaves. When grown with 25 µM berberine, true leaves were not seen on 17% of wild type, 27% of as1-1, and 34% of as2-1 plants (Table 1). These results indicated that as1-1 and as2-1 mutant plants were more sensitive than the wild-type plants to berberine treatment.

Since trumpet-like and filamentous leaves are often generated in plants having defective adaxial–abaxial polarity determination, we examined whether these leaf phenotypes are associated with transcript levels of certain genes in the presence of berberine treatment. To examine the effect of berberine treatment on leaf adaxial–abaxial polarity, we used green fluorescent protein (GFP) under the control of the FILAMENTOUS FLOWER (FIL) promoter (FILp:GFP), which is expressed in abaxial cells of leaf primordia (Watanabe and Okada 2003). Strong signals were detected in cells located at peripheral positions in filamentous leaves in the presence of 20 µM berberine (Fig. 2Bc, Bd), whereas no signals were detected in cells located on the adaxial side of leaves of as1-1 and as2-1 plants in the absence of berberine (Fig. 2Ba, Bb).

We next examined the accumulated transcript levels of genes involved in leaf adaxial–abaxial polarity and class 1 KNOX family genes in 14-day-old shoots treated with 12.5 µM berberine. Figure 2C shows the changes in the accumulated transcript levels of representative genes. Supplementary Table S2 shows relative changes in transcript levels of all the genes analyzed in each plant. When the wild-type plants were treated with 12.5 µM berberine, the accumulated transcript levels of BP and KAN4 showed significant increases over twofold higher than those levels of the non-treated wild type (Fig. 2C). Accumulated transcript levels of BP, KAN4, ETT/ARF3, and ARF4 in either the as1-1 or as2-1 or both plants treated with berberine at 12.5 µM showed significant increases over twofold higher than those of non-treated as1-1 or as2-1 plants (Fig. 2C; Supplementary Table S2). In contrast, the accumulated transcript levels of the adaxial determinant HD-ZIPIII genes PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) were not significantly changed by berberine treatment in wild-type, as1-1, and as2-1 plants (Supplementary Table S2).

Phenotypes of plants treated with other isoquinoline alkaloid compounds

To understand which structure of the isoquinoline alkaloid compounds is responsible for their function, we next examined whether two other isoquinoline alkaloid compounds, coptisine and palmatine, having chemical structures similar to that of berberine (Fig. 3Aa, Ba), affect the phenotypes of as1 and as2 leaves. The sensitivity of plants to coptisine was similar to that of berberine (Fig. 3Ab). Trumpet-like and filamentous leaves were observed at concentrations from 20 to 50 µM in as1-1 and as2-1 plants (Fig. 3Ce, 3Cf), whereas these abnormal leaves were not
observed in wild-type plants (Fig. 3Cd; Table 2). Trumpet-like and filamentous leaves were observed in 28 % (27 out of 96 plants) and 43 % (42 out of 96 plants) of as1-1 and as2-1 plants at 40 µM coptisine, respectively (Table 2).

Plants without true leaves were not observed with coptisine treatment, although growth was inhibited. In contrast, treatments with 100 µM palmatine had no effect on either growth or leaf morphology (Fig. 3Bb, Cg, Ch, Ci). These results suggested that some specific conformation or residues might be important for affecting plant growth and morphology.

Phenotypes of plants treated with a DNA intercalator

Since it has been reported that berberine forms complexes with duplex, triplex, and quadruplex DNA (Klimek and Hnilica 1959; Yamagishi 1962; Kley and Hahn 1969; Lee et al. 1993; Ren and Chaires 1999; Franceschin et al. 2006), we attempted to treat as1 and as2 mutant plants with one of the DNA intercalators, N-methylmesoporphyrin IX (NMM), which has been known as a major quadruplex DNA ligand (Arthanari et al. 1998). NMM treatment severely inhibited growth of plants at 10 µM and plants with red, autofluorescence. Scale bars = 100 µm. C Changes in transcription levels of genes involved in the maintenance of shoot apical meristem and leaf adaxial–abaxial polarity in A. thaliana. RNA was extracted from 14-day-old shoots of WT, as1-1, or as2-1 plants. Each value was normalized by reference to the level of TUA3 transcripts. Error bars show standard deviations (n = 3). Values indicated by dashed lines are shown relative to the values for WT plants treated with 0 µM berberine. An asterisk indicates over a twofold significant increase from each plant not treated with berberine (p < 0.01 by Student’s T test).
**Fig. 3** Phenotypes of Col-0 (WT), as1-1, and as2-1 mutants of A. thaliana in the presence of the isoquinoline alkaloids coptisine and palmatine.  
A Chemical structure of coptisine (a) and gross morphology of WT, as1-1, and as2-1 plants grown on MS medium without and with coptisine for 21 days (b).  
B Chemical structure of palmatine (a) and gross morphology of WT, as1-1, and as2-1 plants grown on MS medium without and with palmatine for 18 days (b).  
C Leaf phenotypes of plants grown without (a, b, c) and with 30 µM coptisine (d, e, f) and 100 µM palmatine (g, h, i) for 18 days. (a, d, g) WT plants, (b, e, h) as1-1 plants, (c, f, i) as2-1 plants. Red arrowheads in panels (e, f) show filamentous leaves. Scale bars = 1 mm.

### Table 2 Frequencies of plants with abnormal leaves and lethal phenotypes after growth on medium that contained coptisine

| Coptisine (µM) | Number of plants with pointed leaves | Number of plants with trumpet-like and filamentous leaves | Number of plants without true leaves |
|----------------|-------------------------------------|----------------------------------------------------------|-------------------------------------|
| Col-0 (WT)     | 0 0 0 0 0 0 0 0 0 1 0 0 0 0 3 2      |                                                          |                                     |
| as1-1          | 0 1 0 1 1 1 0 8 16 21 27 21 0 1 0 0 7 29 |
| as2-1          | 0 2 0 3 1 0 0 16 37 42 42 37 0 1 1 3 11 30 |

Plants (n = 96) were used for each coptisine treatment.
The filamentous or trumpet-like leaves on each plant were counted under the stereomicroscope at 18 days after sowing.
rarely germinated at 25 µM (Fig. 4Ab). NMM treatment strongly inhibited true leaf formation. As shown in Fig. 4C, the sensitivity to NMM was stronger in as1-1 and as2-1 plants than in wild-type plants: 73 % of as1-1 plants and 75 % of as2-1 plants were deficient in true leaf formation, whereas 61 % of the wild-type plants were deficient at 3 µM NMM. Plants treated with NMM showed filamentous leaves in all wild-type, as1-1, and as2-1 plants at low concentrations of NMM; although we did not detect a significant difference in the frequencies of filamentous leaf formation between wild-type and the as1-1 or as2-1 mutant plants (Fig. 4Bd, Be, Bf).

Increased sensitivity of berberine by a mutation in cytosolic ribosomal protein RPL5B

Most DNA intercalators are also known to bind to ribosomal RNA, a component of the ribosome complex, and result in ribosome degradation (Wolfe et al. 1972). We treated wild-type, oli7-1, and oli7-1 as2-1 double mutant plants with berberine. Berberine treatment severely inhibited the growth of oli7-1 plants at 15 and 20 µM (Fig. 5A). When we treated these plants with berberine, 4 and 18 % of oli7-1 plants did not have true leaves at 15 and 20 µM berberine, respectively, whereas very lower frequencies (1
and 2%) of wild-type plants appeared without true leaves at 15 and 20 µM berberine (Fig. 5B). The oli7-1 plants treated with 20 µM berberine also displayed the narrower leaf phenotype (orange arrowheads in Fig. 5Ag). When treated with 15 and 20 µM berberine, the narrow-leaf phenotype was found in 4 and 1% of oli7-1 plants, 34 and 29% of oli7-1 as2-1 double mutant plants, and 1 and 3% of wild-type plants, respectively (Fig. 5A, B). Trumpet-like and filamentous leaves were also observed at 15 and 20 µM berberine treatment in 5 and 1% of oli7-1 plants, respectively, which are comparable frequencies to those of wild-type plants. As reported previously (Horiguchi et al. 2011a), 17% of the oli7-1 as2-1 double mutants displayed a filamentous-leaves phenotype in the absence of berberine, and the frequencies of the filamentous-leaves phenotype were approximately twofold increased (37%) in the presence of both 15 and 20 µM berberine.

The effects of berberine on transcript levels in wild-type plants

To know how berberine affects leaf cells in wild-type plants, we used Affymetrix ATH1 microarrays to analyze transcript levels of genes in Arabidopsis plants grown on MS medium with and without 12.5 µM berberine for 14 days. Microarray analysis showed that transcript levels of 3,854 out of 22,591 genes on the ATH1 gene chip (excluding the mitochondrial genome) were changed by berberine treatment (Supplementary Table S3). To date, mutations of many ribosomal protein genes and other genes have been identified that enhance as1 and or as2 leaf phenotypes (Ori et al. 2000; Xu et al. 2003, 2006; Kidner and Martienssen 2005; Li et al. 2005; Garcia et al. 2006; Huang et al. 2006; Yang et al. 2006; Ueno et al. 2007; Pinon et al. 2008; Yao et al. 2008; Pérez-Pérez et al. 2010; Horiguchi et al. 2011a, b; Kojima et al. 2011; Szakonyi and Byrne 2011; Ishibashi et al. 2012). We analyzed whether the expression of these known enhancer genes was repressed by berberine treatment; however, transcript levels of such genes were not changed significantly by berberine.

Discussion

In the present study, we found that berberine affected the establishment of leaf polarity in the as1 or as2 mutant plants of A. thaliana. Our results by treatments with low molecular weight compounds implicated the existence of a novel factor(s), which is involved together with AS1 and AS2 in the establishment of adaxial cells of leaves.
Berberine affects leaf development of Arabidopsis and upregulates the transcript levels of class 1 KNOX and leaf abaxial determinant genes

When we treated the as1-1 and as2-1 mutant plants with berberine, they produced abaxialized filamentous leaves (Fig. 2A). Berberine might inhibit a function(s) of a novel factor(s) that is involved in adaxialization independently from AS1–AS2 during leaf development. Namely, the factor(s) and AS1–AS2 might share the same target to control the adaxialization of leaves through independent pathways (Fig. 6). It has been reported that the AS1–AS2 complex directly represses the transcription of BP and KNAT2 genes (Guo et al. 2008). Transcription of the abaxial determinant genes ETT/ARF3, KAN2, and YAB5 was also repressed by AS1 and AS2 (Iwakawa et al. 2007). Our real-time qRT-PCR results also showed that berberine treatment increased transcript levels of BP and KAN4 genes in wild-type plants, and the class 1 KNOX genes BP and KNAT6 and the abaxial determinant genes KAN4, ETT/ARF3, and ARF4 in the as1 and as2 mutants. These results suggest that the novel factor(s) inhibited by berberine somehow represses BP and KAN4 genes through an unidentified pathway that would act in parallel with AS1–AS2 for the establishment of leaf polarity, resulting in the formation of flat symmetric leaves in A. thaliana (Fig. 6).

Factors inhibited by berberine and coptisine that are required for the establishment of leaf polarity in parallel with AS1 and AS2

Berberine, coptisine, and palmatine are close structural analogs of each other. They bear the same tetracyclic structure (7,8,13,13a-tetrahydroberberinium), but differ in the nature of the substituents at positions 2 and 3 on the benzo ring, being methylene dioxy for berberine and coptisine, and dimethoxy for palmatine. Similarly to berberine, coptisine and palmatine also have a buckled structure. Recently, berberine and coptisine have been reported to inhibit the receptor activator of nuclear factor-kappaB ligand (RANKL)-induced osteoclast differentiation from osteoclast precursors, the survival of mature osteoclasts, and ovariectomy-stimulated bone resorption in rats (Li et al. 1999; Hu et al. 2008; Lee et al. 2008; Lee et al. 2012). In contrast, the inhibitory effect of palmatine on osteoclast differentiation and function is markedly weaker than that of berberine (Hu et al. 2008; Lee et al. 2010, 2012). Since both berberine and coptisine affected leaf development, the methylene dioxy substituent at positions 2 and 3 on the benzo ring might be responsible for determining their targets, although their targets might be different from that in RANKL-induced osteoclast differentiation in rats.

Multiple pathways are involved in the adaxial development of as1 and as2 leaves

Our microarray analysis showed that berberine negatively affected the expression of many genes; however, we did not detect decreased transcript levels of those genes that have been known to interact genetically with AS1 and/or AS2. To seek for the means by which berberine repressed such unknown factor(s), we considered several possible mechanisms of repression (inhibition), such as via berberine intercalation with secondary structures of DNA (quadruplex and triplex) and RNA, or by binding with proteins (Meyerson et al. 1978). Berberine treated plants having double mutations of AS2 and RPL5B showed more severe defects in polarity than did those as2 single mutant plants (Fig. 5A, B). These results suggest that berberine might repress other ribosomal components in the same pathway with RPL5B/OLI7/ANG3. Our genetic analysis also suggests that a process mediated by an unknown factor(s), whose function is inhibited by berberine, is the distinct process involving RPL5B for the establishment of leaf polarity. As we previously suggested in Kojima et al. (2011), the adaxial development of leaves might be controlled independently by multiple pathways that include the products of ELO3, RDR6-AGO7, and HDT1/HDT2. Considering that plants treated with another G-quadruplex ligand NMM also showed filamentous leaves (Fig. 4B), it might also be possible that berberine represses unknown factors via intercalating secondary DNA/RNA structures such as G-quadruplex DNAs. Especially, a DNA sequence that putatively forms a G-quadruplex is found in the transcription initiation site of ribosomal DNA in both the human (Hanakahi et al. 1999) and Arabidopsis genomes (Takahashi et al. 2012). It remains to be analyzed whether berberine binds to the rDNA region or to rRNA with the resulting impaired formation of leaf polarity. Taken
together, the adaxial development of leaves is likely to be controlled independently by multiple pathways on the as1 or as2 background.

Since adaxial defects of leaves caused by berberine are not obvious in wild-type plants (Fig. 2C), the wild-type AS1–AS2 function can replace the polarity establishment function of an unknown factor(s) that is inhibited by berberine. That is to say, the wild-type AS1 and AS2 genes appear to attenuate deficiencies resulting from treatments with berberine and coptisine. Further investigations of the molecular functions of AS1 and AS2 proteins will allow us to understand how they could regulate gene expression by the presently unknown mechanism(s).

Our experiments are representative of a new method to identify novel factors that take part in leaf development by using the as1 or as2 mutant. The combination of comprehensive chemical screening and genetic analysis may make it possible to elucidate new events involved in the establishment of leaf polarity.

**Materials and methods**

**Plant strains and growth conditions**

*Arabidopsis thaliana* ecotype Col-0 (CS1092) and the mutants as1-1 (CS3374) and as2-1 (CS3117) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Columbus, OH, USA). We outcrossed as2-1 with Col-0 three times and as1-1 with Col-0 once and used the progeny for our experiments (Semiarti et al. 2001). The oli mutants, back-crossed to Col-0 more than three times, were used for our experiments (Horiguchi et al. 2006; Fujikura et al. 2009). For the analysis of plants, seeds were surface-sterilized with 0.25 % sodium hypochlorite/0.1 % Triton X-100 solution and sown on Murashige and Skoog (MS) medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 2 % (w/v) sucrose and 0.8 % agar. After 2 days at 4 °C in darkness, plants were transferred to a daily regimen of 8 h of darkness and 16 h of white light at 50 μmol m⁻² S⁻¹ at 22 °C, as described previously (Semiarti et al. 2001). Berberine chloride and palmatine chloride were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Coptisine chloride was purchased from Frontier Scientific, Inc. (Logan, Utah, USA). For chemical treatments, berberine, coptisine, or palmatine dissolved in dimethyl sulfoxide, or NMM dissolved in H₂O was mixed with MS agar medium at 1–100 μM concentrations and immediately dispensed into plastic multi-dishes (multidish 267061, Thermo Fisher Scientific K. K., Yokohama, Japan). Ages of plants are given in terms of the number of days after sowing.

**Morphological observation of leaves**

Whole leaves were observed under a stereoscopic microscope (Stemi SV11, Carl Zeiss Inc., Oberkochen, Germany). Leaves were fixed in a formalin-acetic acid-alcohol solution and cleared in a chloral hydrate solution as previously described (Tsuge et al. 1996).

**Quantification of angles of leaf tips**

First or second true leaves were dissected from seedlings treated with and without berberine, mounted on a glass slide, and photographed under a stereoscopic microscope (Stemi SV11, Carl Zeiss). We drew a line along the mid vein from the one-eighth distal area of a leaf blade and a vertical line on leaves of the digital image using Adobe Illustrator software (Adobe Systems Incorporated, San Jose, CA, USA). We then drew lines toward the leaf tips from the crossing points with the vertical line and the outline of the leaf blade. The resulting angles were calculated by using the software ImageJ (http://rsbweb.nih.gov/ij/). Statistical significance was calculated by Student’s T test and F test by using Excel (Microsoft Corporation, Tokyo, Japan).

**Histological analyses**

Thin sections and cleared specimens were prepared as described previously (Semiarti et al. 2001). Leaf sections were cut by hand, and GFP fluorescence images were obtained with a confocal laser microscope (LSM510, Carl Zeiss Inc., Oberkochen, Germany) according to Ueno et al. (2007).

**Real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)**

Shoots were harvested 14 days after seeds were sown and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from 14-day-old seedlings with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Sample volumes were normalized as described by Iwakawa et al. (2007). The primer pairs are shown in Supplementary Table S1. For the analysis of RNA levels in Arabidopsis by real-time qRT-PCR, we prepared 10 μg of total RNA. Reverse transcription was carried out with ReverTra Ace (TOYOBO, Osaka, Japan). PCR was performed in the presence of the double-stranded DNA-specific dye SYBR Green (Applied Biosystems, Lincoln, CA). Amplification was monitored in real time with the Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems) according to the supplier’s recommendations. The mean
value of three technical replicates was normalized by using the TUBULIN α-3 (TUA3) transcript as a control. Statistical significance was calculated by Student’s T test and F test by using Excel.

Microarray

Seedlings of wild-type plants grown with and without 12.5 μM berberine for 14 days were harvested and total RNA was extracted as described in the ‘real time qRT-PCR’ section, above. For microarray analysis, the quality and purity of the RNA were confirmed with an Ultrospec 2100 pro (GE Healthcare UK Ltd, Amersham Place, England). Total RNA samples (8 μg) were reverse-transcribed, yielding double-stranded cDNA, which was transcribed in vitro in the presence of biotin-labeled nucleotides with an IVT Labeling Kit (Affymetrix Inc., Santa Clara, CA), and purified. Labeled cRNA was fragmented and hybridized to Affymetrix ATH1 GeneChip arrays for 16 h at 45 °C according to Affymetrix protocols. Arrays were washed on an Affymetrix Fluidics Station 450 and measured for fluorescence intensity with an Affymetrix GeneChip Scanner 3000. The raw data were processed by using Affymetrix Gene Chip Operating Software (GCOS; Version 1.4.0.036).

Microarray analysis

We calculated the expression signals for two samples and the log2 ratio for berberine-treated samples against the control sample by GCOS. The detection calls and change calls were calculated based on the signal-intensity ratio between perfect-match and miss-match oligos on the array. In this experiment, we excluded those genes (6,782 genes) for which two samples showed an absent or marginal call (i.e., detection call determined by GCOS: no call), because no change indicates that the expression signal was undetectable. We also excluded those genes (18,737 genes) for which two samples showed no change calls (i.e., change call determined by GCOS: no change call means 1–0.006 ≥ p ≥ 0.006 for the one-sided Wilcoxon signed-rank test), because no change indicates that the expression signal is almost equal to that of the control sample.

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