

Analysis of the expression of *BohLOL1*, which encodes an LSD1-like zinc finger protein in *Bambusa oldhamii*

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Abstract A cDNA, *BohLOL1*, encoding a protein containing three zf-LSD1 (zinc finger-Lesions Simulating Disease resistance 1) domains was cloned from growing bamboo (*Bambusa oldhamii*) shoots. A phylogenetic analysis revealed that *BohLOL1* is a homolog of *Arabidopsis* LSD1 and LOL1 (LSD-one-like 1), which have been reported to act antagonistically in controlling cell death via the maintenance of reactive oxygen species homeostasis. The *BohLOL1* gene was differentially expressed in various bamboo shoot tissues and was upregulated in shoots with higher rates of culm elongation. The expression level of this gene in multiple shoots of bamboo, which were cultured in vitro, was also upregulated by auxins, cytokinins, pathogen infection, 2,6-dichloroisonicotinic acid (a functional analog of salicylic acid), and hydrogen peroxide. The results suggest that *BohLOL1* participates in bamboo growth and in the response to biotic stress. The DNA-binding assays and subcellular localization studies demonstrated that *BohLOL1* is a nuclear DNA-binding protein. *BohLOL1* might function through protein-DNA interactions and thus affect the expression of its target genes. The results of this study extend the role of plant LSD1 and LOL1 proteins from the regulation of cell death to cell growth. The growth-dependent up-regulation of *BohLOL1* expression, which uniquely occurs in growing bamboo, might be one of the critical factors that contribute to the rapid growth of this remarkable plant.

Keywords Bamboo growth · *Bambusa* · Disease resistance · DNA-binding protein · LSD-one-like 1 · Nuclear localization · Zf-LSD1 domain

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzylaminopurine
CuZnSOD	Copper zinc superoxide dismutase
DD RT-PCR	Differential display reverse transcription-polymerase chain reaction
HR	Hypersensitive response
IAA	Indole-3-acetic acid
IMAC	Immobilized metal affinity chromatography
INA	2,6-Dichloroisonicotinic acid
LSD1	Lesions simulating disease resistance 1
LOL1	LSD-one-like 1
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
PMSF	Phenylmethanesulfonyl fluoride
rcd	Runaway cell death
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
TDZ	Thidiazuron
zf-LSD1	Zinc finger-LSD1

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Introduction

LSD1 genes in plants were first defined from several *Arabidopsis* *lsd* (lesions simulating disease resistance) mutants that developed hypersensitive response (HR)-like necrotic lesions in the absence of pathogens (Dietrich et al. 1994,

1997). The initiation of necrotic lesions in the *lsd1* mutant can be triggered by pathogens, chemical inducers of systemic acquired resistance (SAR) or long-day growth conditions, after which the lesions spread unchecked beyond the site of initiation and give rise to the runaway cell death (*rcd*) phenotype of this mutant (Dietrich et al. 1994). The superoxide generated in the apoplast was determined as sufficient to trigger lesions in the *lsd1* mutant; the role of LSD1 in setting the local boundaries of cell death during an HR was proposed to occur through monitoring of a superoxide-dependent signal and negatively regulating a plant cell death pathway (Jabs et al. 1996). Another study showed that LSD1 is part of a signaling pathway for the induction of copper zinc superoxide dismutases (CuZn-SOD) in response to salicylic acid (SA). Induction of the CuZnSOD proteins, which catalyze the conversion of superoxide to hydrogen peroxide, decreases the levels of superoxide that accumulates during an HR, suggesting a role for LSD1 as a regulator of reactive oxygen species (ROS) levels (Kliebenstein et al. 1999). In addition, SA is both necessary and sufficient for *rcd* in the *lsd1* mutant, and LSD1 can perceive and control the spread of an SA-dependent signal leading to cell death (Aviv et al. 2002). Moreover, LSD1 is required for acclimation to conditions that promote photooxidative stress (Dietrich et al. 1997; Mateo et al. 2004; Torres et al. 2005; Mühlenbock et al. 2008) and is involved in the control of lysigenous aerenchyma formation, a consequence of programmed cell death and cell wall autolysis, in *Arabidopsis* hypocotyls under root hypoxia (Mühlenbock et al. 2007). Thus, LSD1 has been proposed as one of the key homeostatic regulators of a global acclimatory and defense mechanism in plants (Mühlenbock et al. 2008).

The deduced amino acid sequence of *Arabidopsis* *LSD1* (termed *AtLSD1* below) contains three C2C2 zinc finger domains, CxxCxRxxLMYxxGASxVxCxxC, which were termed the zf-LSD1 domain (Dietrich et al. 1997). Using cDNA cloning or database searches with the sequence of the zf-LSD1 domain, *LSD1*-like genes have been identified in *Arabidopsis*, rice and other species (Epple et al. 2003; Coupe et al. 2004; Wang et al. 2005; Xu and He 2007; Liu and Xue 2007). These genes can be classified into three major classes according to the copy number (one to three) of the zf-LSD1 domain present; however, there is no essential homology among the three classes, except in the zinc-finger domains (Liu and Xue 2007). In the *Arabidopsis* genome, five *LSD1*-like genes are present. Among them, *AtLOL1* (*LSD-one-like 1*) contains three copies of the zf-LSD1 domain, as does *AtLSD1*. In contrast to the function of *AtLSD1*, *AtLOL1* is a positive regulator of PCD, and these two proteins act antagonistically to regulate oxidative stress-induced cell death (Epple et al. 2003). In rice, *OsLSD1* is a functional homolog of *AtLSD1*;

however, it shows a higher sequence homology to *AtLOL1* than to *AtLSD1* (Wang et al. 2005). In addition to being a negative regulator of cell death, *OsLSD1* also plays a positive role in callus differentiation (Wang et al. 2005). The functional differences between *AtLOL1* and *OsLSD1* can be attributed to the differences in posttranslational modifications or to differences in their N- and C-termini, as suggested by Wang et al. (2005) and Liu and Xue (2007).

Bamboo, which belongs to the subfamily Bambusoideae of the family Poaceae, is distinguished by its remarkably rapid growth; however, little is known about the corresponding regulatory mechanism. To identify genes that are involved in the rapid growth of bamboo, we performed differential display RT-PCR (DD RT-PCR) and isolated hundreds of expressed sequence tags (ESTs) that were differentially expressed in shoots of *Bambusa oldhamii* at different growth stages. Among the isolated ESTs, an *LSD1*-like gene (designated *BohLOL1*) was unexpectedly identified. To understand why bamboo requires an *LSD1*-like gene to be expressed in rapidly growing shoots, in this study, we analyzed the expression of *BohLOL1* in bamboo and characterized the biochemical identity of the protein encoded by the gene. Our results show that *BohLOL1* not only is involved in the response to pathogen infection but also may play a role in the regulation of growth. The function of *BohLOL1* could be exerted via its DNA-binding activity to affect the expression of target genes that participate in disease resistance or plant growth.

Materials and methods

Plant materials

Etiolated shoots (average height: 15 cm) that had not yet emerged from the ground and green shoots (average height: 100 cm) of *Bambusa oldhamii* Murno were collected from a bamboo farm in Taipei, Taiwan, as previously described (Chiu et al. 2006). Each shoot was divided into three parts: the base, which is the area 5–10 cm above the point where the shoot emerges from the node of the pseudorhizomes; the middle, which consists of the developing nodes and internodes; and the top, which consists of the shoot meristem and a series of overlapping sheaths surrounding the developing nodes and internodes. The samples were immediately frozen in liquid nitrogen and stored at -80°C for later use.

Multiple shoots of *B. oldhamii* cultured in vitro were maintained in MS medium supplemented with $0.45\ \mu\text{M}$ thidiazuron (TDZ; Sigma–Aldrich, St. Louis, MO, USA) at 25°C , as previously described (Lin et al. 2007). For studying the effects of biotic and abiotic factors on *BohLOL1* expression, multiple shoots were transferred to fresh

media containing TDZ and cultured for 14 days. The shoots were then infected with a pathogen, treated with 2,6-dichloroisonicotinic acid (INA, Sigma) or hydrogen peroxide (H₂O₂) or grown at different temperatures, which were performed as follows:

Pathogen infection: *Scolecobasidium longiphorum* Matsushima (BCRC 33216), which was isolated from rotten culms of bamboo in Taiwan, was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Mycelia of this fungus was grown for 7 days in a corn meal agar plate (5% (w/v) corn meal, 1.5% (w/v) agar), after which a 1-cm² agar plug containing the culture was excised from the plate and then added to 25 mL of potato dextrose broth (0.4% (w/v) potato starch, 2% (w/v) dextrose); the mycelia was then grown at 25°C for 3 days. Multiple shoots of bamboo were immersed in the liquid culture of the fungus for 1 min and then transferred to MS agar medium supplemented with 0.45 μM thidiazuron. The shoots were incubated at 25°C for different time periods. Controls (without pathogen infection) were incubated under the same conditions for all time points.

2,6-Dichloroisonicotinic acid or H₂O₂ treatment: 2,6-dichloroisonicotinic acid (0.05 mM) or H₂O₂ (10 mM) was added to the growth medium of the multiple shoots of bamboo, after which the shoots were incubated at 25°C in the dark. The shoots were collected at different time points. Controls (without INA or H₂O₂) were incubated at the same conditions for all time points.

Temperature effect: The multiple shoots were placed at 4, 25, 37 or 42°C in the dark and then collected at different time points.

For testing the effects of cytokinins and auxins, the multiple shoots of bamboo were incubated with fresh media without TDZ for 14 days. Then 1-naphthaleneacetic acid (NAA; Sigma), indole-3-acetic acid (IAA; Sigma), 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), kinetin (Sigma) or 6-benzylaminopurine (BA; Sigma) was added to the medium at various concentrations. The shoots were then incubated at 25°C in the dark for 12 h.

Cloning of *BohLOL1* cDNA and sequence analysis

Several clones, which were selected from an EST library of bamboo constructed previously in our laboratory by DD RT-PCR and putatively encoded proteins involved in transcriptional regulation, were subjected to reverse northern analysis (data not shown). One clone (designated *BohLOL1*) that exhibited an up-regulated expression pattern in the rapidly elongating bamboo culms was selected for full-length sequencing. The sequence alignment was performed using the program CLUSTAL W (Thompson et al. 1994). A phylogenetic tree of the alignment based on

the amino acid sequences was generated using the program Phylogendron (version 0.8d, beta) (Gilbert 1999).

Real-time RT-PCR

Total RNA was isolated from different bamboo samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized as previously described (Chen et al. 2010) and used as a template for real-time PCR with primers specific for *BohLOL1* (forward primer: 5'-GGCAGCTTCTCAAAGGATG-3'; reverse primer: 5'-CAAGTCAACGAATTTGAGCAC-3') or *BohUBQ5* (forward primer: 5'-ACCACTTCGACCGCCACTACT-3'; reverse primer: 5'-AGCAGCCTACGCCTTCTGGTT-3'). The PCR reactions were performed using the SYBR FAST qPCR Kit (KAPA BIOSYSTEMS, Boston, MA, USA) in an ABI Prism 7000 real-time thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Each PCR reaction was run in triplicate, and no-template controls were included for each primer pair. At the end of the PCR reaction, a dissociation curve analysis was performed to assess the specificity of the amplification. The amplified products were further confirmed by gel electrophoresis and DNA sequencing. *BohUBQ5*, which encodes a ubiquitin in bamboo, was used as the reference gene; the stability of its expression across different bamboo samples was previously validated using the method of Brunner et al. (2004) (Chen CY and Wang AY, unpublished data). The abundance of the *BohLOL1* transcript in each sample was determined by normalization to *BohUBQ5* using the formula $2^{-[Ct(BohLOL1) - Ct(BohUBQ5)]}$. The fold-changes in the abundance of the *BohLOL1* transcript between various samples were determined using the $\Delta\Delta Ct$ method (Bookout et al. 2006).

Expression and purification of recombinant *BohLOL1* from *E. coli*

The coding region of *BohLOL1* was amplified by PCR with the primers 5'-GGATCCATGCCGGTTCCCCTTGC-3' and 5'-TCAGTTGCTGGGCTTCTGGTCTGC-3' and with *BohLOL1* cDNA as the template. The amplified DNA was cloned into the pGEM-TEasy (Promega, Madison, WI, USA) by TA cloning, yielding pGEM-LOL1. The coding region of *BohLOL1* was cut from the pGEM-LOL1 plasmid and subcloned into the *Bam*HI and *Pst*I sites of pTrcHisA (Invitrogen). The resulting plasmid, pTH-*BohLOL1*, was transformed into *E. coli* TOP10 cells for expression. The production of His-tagged recombinant *BohLOL1* proteins was induced at the mid-log phase by adding IPTG to a final concentration of 1 mM. After growing at 37°C for 5 h, the cells were harvested by centrifugation, washed with PBS (10 mM sodium phosphate, pH 7.0, 130 mM NaCl),

resuspended in PBS, disrupted by sonication and then centrifuged. The recombinant BohLOL1 proteins in the supernatant were purified by cobalt-based immobilized metal affinity chromatography (IMAC). The column was first washed with buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0) containing 10 mM imidazole, and then the proteins that bound to the column were eluted with the same buffer containing 150 mM imidazole. Proteins in the eluted fractions were subjected to SDS-PAGE and western analysis with an anti-(His)₆ antibody (GE, Piscataway, NJ, USA). The fractions containing His-tagged BohLOL1 were pooled, concentrated and desalted by ultrafiltration (Amicon Centriplus YM-3, Millipore, Billerica, MA, USA). The concentrated recombinant proteins were used in a filter-binding assay and southwestern analysis. For raising polyclonal antibodies against BohLOL1, the concentrated proteins were separated on SDS-PAGE, and the protein band of the recombinant BohLOL1 was cut from the gel and used to immunize BALB/c mice.

DNA-binding assays

The genomic DNA that was isolated from the etiolated bamboo shoots using the Genomic DNA Mini Kit (Geneaid, Taipei, Taiwan) was sheared to obtain fragments ranging from 100 to 500 bp by sonication. These DNA fragments were ³²P-labeled using the Amersham Rediprime II Random Priming Labelling System (GE). The recombinant BohLOL1, purified by cobalt-based IMAC, was mixed with the double-stranded, ³²P-labeled genomic DNA fragments, and the filter-binding assay was conducted according to the method described by Papoulas (2001).

For the southwestern analysis, recombinant BohLOL1 proteins were separated on 13.3% tricine SDS-PAGE (Schagger and Vonjagow 1987) and then transferred to a PVDF membrane (Millipore). The membrane was hybridized with the ³²P-labeled genomic DNA fragments according to the method described by Siu et al. (2008). After hybridization, the membranes were exposed to an image plate, and the captured images were analyzed with a Bio Imaging Analyzer BAS1000 (Fujifilm, Tokyo, Japan).

Intracellular localization assays

The coding region of *BohLOL1* was cut from the pGEM-LOL1 plasmid and subcloned into the *Bam*HI and *Not*I sites of pHBT95sGFP (Chiu et al. 1996). The resulting plasmid, which carried the *35S*C4PPDK-*sGFP-BohLOL1* construct, was named pHBT95sGFP-LOL1.

Protoplasts were isolated from the leaves of multiple shoots of *B. oldhamii* using the method for isolating rice protoplasts (Bart et al. 2006); a different enzyme solution

(2% cellulase RS [Yakult, Tokyo, Japan], 1% macerozyme R-10 [Yakult], 5 mM 2-[*N*-morpholino]ethanesulfonic acid [pH 5.6], 0.6 M mannitol, 68 mM CaCl₂·2H₂O and 1% bovine serum albumin) was used to digest the cell walls, and 5 mM glucose was included in the W5 solution for washing the protoplasts. The transformation of the bamboo protoplasts and the confocal laser scanning microscopy analysis were performed as described by Wu et al. (2009). To express a nuclear marker protein, a plasmid carrying *VirD2-NLS-mCherry* (Lee et al. 2008) was co-transferred into the protoplasts with pHBT95sGFP-LOL1 or pHBT95sGFP.

Fractionation of cytoplasmic proteins and nuclear proteins

Bamboo shoots were ground into powder in liquid nitrogen. Three volumes (v/w) of STMDPS buffer (250 mM sucrose, 50 mM Tris-HCl [pH 7.4], 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 25 mg/mL spermine and 25 mg/mL spermidine) (Cox and Emili 2006) were added and mixed. The homogenates were used for the extraction and fractionation of cytoplasmic proteins and nuclear proteins according to the method described by Cox and Emili (2006). Proteins of the cytosolic and nuclear fractions were subjected to 13.3% tricine SDS-PAGE and western analysis. Antibodies against BohLOL1, human histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and sea urchin α -tubulin (Sigma) were used to detect proteins on the membranes. Histone H1 and α -tubulin were used as the nuclear and cytoplasmic markers, respectively.

Statistical analysis

Each experiment was repeated at least three times. Student's *t* test was conducted for data in Figs. 3, 4, 5, 6. The data were considered significantly different when *P* < 0.05.

Results

Identification of bamboo *BohLOL1* cDNA

To investigate the genes involved in bamboo growth, an EST-library was constructed by differential display RT-PCR. Several genes, which encoded transcription regulatory proteins and exhibited differential expression patterns at different development stages, were identified, and their expression patterns were further investigated by reverse northern analysis (data not shown). One of the clones with an up-regulated expression pattern in the elongating culm of bamboo was selected for further study. The BLAST

analysis showed that it was an *LSD1-like* gene, and thus, it was designated *BohLOL1*.

The *BohLOL1* cDNA was 764 bp in length, which included a 5'-UTR, an open reading frame that encoded a 14.7-kDa polypeptide, a 3'-UTR and a poly(A) tail. The deduced amino acid sequence of *BohLOL1* contained 3 zf-LSD1 domains and exhibited high identity with rice *OsLSD1*, maize *ZmLOL1* and *Arabidopsis AtLOL1* (Fig. 1). A phylogenetic analysis of the sequences of LSD1-like proteins containing 2 or 3 zf-LSD1 domains from different plants indicated that *BohLOL1*, *OsLSD1*, *ZmLOL1* and *AtLOL1* were orthologs from different species (Fig. 2).

Expression of *BohLOL1* in growing bamboo shoots

The expression patterns of *BohLOL1* in growing bamboo shoots were further validated by real-time RT-PCR. The transcript levels of *BohLOL1* in the base, middle and top regions of the shoots were all higher in 100-cm green shoots compared with the etiolated shoots, as shown in Fig. 3. The highest level of *BohLOL1* mRNA was observed in the top region of the 100-cm green shoot, and the largest change in *BohLOL1* mRNA level occurred in the middle region of the 100-cm green shoot. According to the growth curve of *B. oldhamii* that was reported by Lin (1958), 100-cm green shoots had higher rates of culm elongation. Therefore, the increase in *BohLOL1* mRNA abundance paralleled the elongation rate.

Effects of biotic and abiotic stress and of phytohormones on the expression of *BohLOL1*

The results described in the previous section suggested that *BohLOL1* expression was related to growth, while its homologs in *Arabidopsis* and rice, *AtLOL1*, *OsLSD1* and *AtLSD1*, are involved in defense and/or acclimatory mechanisms. Therefore, we examined whether *BohLOL1* expression was affected by biotic and abiotic factors and by phytohormones. Because *BohLOL1* expression varied in different regions of bamboo shoots and it is difficult to change the growth conditions of bamboo shoots, which are connected to a clump of mature bamboo plants via extensive subterranean stems, multiple shoots cultured in vitro were used to investigate *BohLOL1* expression under different conditions.

To reveal the effect of pathogen infection on *BohLOL1* expression, multiple shoots were infected with *S. longiphorum*. A clear development of mycelia of this fungus on the multiple shoots was observed after three days of inoculation (data not shown). An analysis of the *BohLOL1* mRNA levels by real-time RT-PCR showed that the pathogen infection resulted in an increased accumulation of *BohLOL1* mRNA after three days of infection (Fig. 4a), which was correlated with the pathogen development. To

investigate the effect of SA, which is a chemical inducer of SAR, multiple shoots were treated with its functional analog, INA. The effect of H₂O₂ was also analyzed because a rapid increase in H₂O₂ and other ROS in the apoplast has been reported as one of the earliest responses observed after pathogen attack, and an increase in the SA level also influences the level of H₂O₂ (reviewed in Shetty et al. 2008 and Vlot et al. 2009). Besides, H₂O₂ is one of the ROS produced in plants during growth and development (Bell et al. 2009; Ros Barceló and Gómez Ros 2009; Swanson and Gilroy 2010). As shown in Fig. 4b, the levels of *BohLOL1* mRNA were increased after 12 h of INA treatment and reached a maximum level at 48 h of treatment. In addition, a dose-dependent effect of INA on *BohLOL1* expression was observed (data not shown). The expression of *BohLOL1* was also enhanced after 12 h of treatment with 10 mM H₂O₂ (Fig. 4c) but decreased slightly in the presence of 200 mM NaCl (data not shown).

We also investigated the effect of temperature on the levels of *BohLOL1* mRNA. *B. oldhamii* is a tropical species; thus, the onset of shooting and the growth of culms occur from summer to autumn (Lin 1961), during which the temperatures are usually higher than 30°C and are very often close to 37°C. Therefore, the multiple shoots of bamboo were incubated at 37°C in addition to 4°C (cold stress), 25°C (the temperature for grown the in vitro cultured multiple shoots) or 42°C (heat stress). At 37°C, significant increases in the levels of *BohLOL1* mRNA were observed after 24 h of incubation (Fig. 5); in contrast, under heat stress, significant decreases in the *BohLOL1* mRNA levels were observed. Under cold stress, the levels of *BohLOL1* mRNA decreased slightly after 24 h of incubation. Figure 6 shows the effects of different auxins and cytokinins on the transcript levels of *BohLOL1*, as determined by real-time RT-PCR. Within 12 h of treatment, each of the auxins employed increased the transcript levels of *BohLOL1*; IAA and 2,4-D showed more significant effects. The application of BA and kinetin also had positive effects on the *BohLOL1* mRNA levels; however, the changes were less than twofold compared to the control.

DNA-binding activity of *BohLOL1*

The presence of zinc-finger domains in *BohLOL1* revealed the possibility that this protein is a DNA-binding protein. To analyze whether *BohLOL1* possessed DNA-binding activity, recombinant His-tagged *BohLOL1* proteins were expressed and purified from *E. coli*. The molecular mass of the recombinant protein was estimated by SDS-PAGE as approximately 19 kDa (Fig. 7a, left and middle panels), which was very close to the predicted size (18.7 kDa). Because the recombinant *BohLOL1* proteins were not purified to homogeneity, *E. coli* cells harboring the

zf-LSD1

	(1)	1	10	20	30	40	50	60	70
AtLOL1	(1)	MPVPLAPYPTPPAPALAPS	YNTPPPANGSTSG	QSQLVCS	GCRNLLMYF	VGATS	SVCCAVC	NAVTA	VAVPPP---
BohLOL1	(1)	MPVPLAPHPTP-VPFTFP	-----NGA	QSQLICS	GCRNLLMYS	SAGATS	SVCCAVCS	TMTAV	FAP---
OsLSD1	(1)	MPVPLAPYPTPPVPFTFP	-----NGA	QSQLVCS	GCRNLLMYF	FAGATS	SVCCAVCS	TVTAV	FAP---
ZmLOL1	(1)	MPVPLAPYPAPAPFTFP	-----NGA	QSQLVCT	GCRNLLMYP	PAGATS	SVCCAVCS	TVTAV	FAP---
AtLSD1	(1)	-----MKVAD	MQDQLVCH	GCRNLLMY	FRGAS	NVRCAL	NTINM	VFP	---P
BoLSD1	(1)	-----	MQDQLVCH	GCRNTLMY	PRGATN	NVRCAL	CHI	VNM	VFLHPPH
BoLSD2	(1)	-----	MQDQLMCH	GCRNTLLY	PRGATN	NVRCAL	NTINM	VFL	HPPP
Pt-EEE85297	(1)	-----	MQSQVVC	RGCASV	LLYPS	GASNVCC	ALCST	TVT	SIFSP---
Pt-EEE97678	(1)	-----	MQSQVVC	RGCSSL	LLYPS	GATNVCC	ALCST	TVT	SIFAP---
Os-BAG99311	(1)	-----	MQSQIVCH	GCRSVL	RYES	GAPSVCC	ALCQA	IT	VFPFA--
Zm-CBC44879	(1)	-----	MQNQIM	CHACRT	LLYPR	GASSVCC	AVCQA	IT	VFPFG--
Os-BAG99442	(1)	-----	MQSQIVCH	GCRNILLY	PRGAP	SVCCAV	CHAV	S	TAFS--
Zm-ACR35025	(1)	-----MFRCLAGW	MRRVRRS	VERSNR	ADMQS	QIVCH	GCR	LLF	YPRGAPSVCCAVCHVVTN
Os-BAH00023	(1)	-----	MQDQLICS	GCRRVV	QYRR	VAGVCC	PCG	NT	LTAVNES
AtLOL2	(1)	-----MEEIQQTQ	KKEEQKH	REFFEE	EEFGPP	GWESA	VI	PP	IVTITAVNPNPTT
Os-BAB93279	(1)	-----MSQLPLAS	QATTDLV	STTAM	QPS	EGIV	DES	LQ	QHPPE

zf-LSD1

	(71)	71	80	90	100	110	120	130	140
AtLOL1	(68)	-----	GTEMAQLV	CGGCH	TLLMY	IRGATS	VQCSC	CHTVN	LALAE-----
BohLOL1	(57)	-----	GTEMAQLV	CGGCH	TLLMY	IRGATS	VQCSC	CHTVN	LALAME-----
OsLSD1	(58)	-----	GTEMAQLV	CGGCH	TLLMY	IRGATS	VQCSC	CHTVN	LALAME-----
ZmLOL1	(58)	-----	GTEMAQLV	CGGCH	TLLMY	IRGATS	VQCSC	CHTVN	LALAME-----
AtLSD1	(44)	PP-----	H--DMAHI	ICGGC	RTMLMY	TRGAS	SVRCS	CCQ	TNLVPAHSNQVAHAP
BoLSD1	(41)	PPPPPP	PHHAHA	GMDMAHI	ICGGC	RTMLMY	TRGAS	SVRCS	CCQ
BoLSD2	(41)	PP-----	PHHAHA	GMDMAHI	ICGGC	RTMLMY	TRGAS	SVRCS	CCQ
Pt-EEE85297	(38)	-----	GMDMAQLI	CRGCR	SLLMY	PHGAT	TVRC	SC	CHV
Pt-EEE97678	(38)	-----	GMDLAQLI	CRGCR	TLLMY	ARGAT	TVRC	SC	CHIV
Os-BAG99311	(39)	-----	PVMEMAH	LICGGC	RTMLMY	TRNAD	TVRC	SC	STV
Zm-CBC44879	(39)	-----	LEMAQLI	CGGCR	TLLMY	TRSA	DTVRC	SC	NTV
Os-BAG99442	(38)	-----	PGMDIA	HLICGGC	RTMLMY	TRNAT	SVRC	SC	CDT
Zm-ACR35025	(60)	-----	PAMEMAR	LICGGC	QTL	MYARS	ATTVRC	SC	CDV
Os-BAH00023	(38)	-----	AVADMSE	LICGGC	PTL	LYNR	GASN	IR	CP
AtLOL2	(57)	-----	EKAQMV	CGSCR	RLSY	LRGSK	HVK	CS	CC
Os-BAB93279	(61)	-SP-----	YLNKE	VGQMV	CGSCR	IL	AYFR	GAY	VH

zf-LSD1

	(141)	141	150	160	170	180	190	200	210
AtLOL1	(117)	MMLLM	QYGAR	SVKCAV	CNFVT	SVGG	STST	TD	SKFNN-----
BohLOL1	(108)	RILLM	QYGAR	SVKCAV	CNFVT	SVGAP	PGAD	QK	PSN-----
OsLSD1	(108)	RMLLM	QYGAR	SVKCAV	CNFVT	SVGAS	PGID	QK	PSS-----
ZmLOL1	(108)	RMLLM	QYGAR	SVKCAV	CNFVT	SVGTS	PGAE	QK	PSS-----
AtLSD1	(104)	RTTLM	YPYGAS	SVKCAV	CQFVT	NVNMS	GRV	FL	TNRPN
BoLSD1	(105)	RTTLM	YPYGAS	SVKCAV	CQFVT	NVNMS	GRV	FL	TNRPN
BoLSD2	(102)	RTTLM	YPYGAS	SVKCAV	CQFVT	NVNMS	GRV	FL	TNRPN
Pt-EEE85297	(88)	RTALM	YPNG	SPSVK	CPVCH	YVTNV	SMA	NRI	PL
Pt-EEE97678	(89)	QTTLM	YPNG	APSVK	CAVCH	YVTN	ISTAN	VR	PL
Os-BAG99311	(90)	RTTLM	YPYGAS	SVKCAI	CHYIT	NTG	MNT	VAPT	PS
Zm-CBC44879	(88)	RTTLM	YPYGAS	SVKCAV	CNYVT	TATG	MNT	VAPT	PS
Os-BAG99442	(89)	QTVLM	YPYGAS	SVKCAI	CHYIT	NTG	MNT	VAPT	PS
Zm-ACR35025	(111)	QTVLM	YPYGAS	SVKCAV	CNEIT	NVGG	VT	PMI	FL
Os-BAH00023	(89)	RTTLM	HEP	PGASTV	QC	ATCR	YV	NHVR	-----
AtLOL2	(105)	KLLLM	YPYGAS	AVRC	SSCNS	VT	DISE	NKR	P
Os-BAB93279	(115)	ATLLM	YPFG	ARAVK	CSL	CL	EV	TEI	GRN

zf-LSD1

	(211)	211	220	230	240
AtLOL1	(155)	-----	-----	-----	-----
BohLOL1	(143)	-----	-----	-----	-----
OsLSD1	(144)	-----	-----	-----	-----
ZmLOL1	(144)	-----	-----	-----	-----
AtLSD1	(161)	TVVV	ENPMSV	DES	GKLVSN
BoLSD1	(165)	TVVV	ENPMSV	NES	GKLVSN
BoLSD2	(164)	TVVV	ENPMSV	NES	GKLVSN
Pt-EEE85297	(146)	TVVV	ENPMSV	DES	GKLVSN
Pt-EEE97678	(151)	TVVV	ENPMSV	DES	GKLVSN
Os-BAG99311	(159)	TVVV	ENPMT	VDEK	GKLVSN
Zm-CBC44879	(147)	TVVV	ENPMT	VDDK	GKLVSN
Os-BAG99442	(143)	TVVV	ENPMT	VDAK	GKLVSN
Zm-ACR35025	(172)	TVVV	ENPMT	VDDK	GKLVSN
Os-BAH00023	(119)	TVLV	ENE	KTLD	DKGKLVSN
AtLOL2	(141)	-QGP---	LKSL	SSLR	RAEN
Os-BAB93279	(149)	-IEQ--	-E	TRTN	SSGL

Fig. 1 Alignment of the deduced amino acid sequences of *LSD1* and *LSD1*-like genes from *B. oldhamii* and other plants. Identical residues are shown on a black background. The C2C2 zinc finger domains (zf-LSD1, CxxCxRxxLMYxxGxSVxCxxC) are indicated by brackets above the sequences. The sequences aligned were from *B. oldhamii* (BohLLO1, GenBank accession number ABW70167), *Oryza sativa* (OsLSD1, AAS13688; Os-BAG99442; Os-BAH00023; Os-BAB93279), *Arabidopsis thaliana* (AtLSD1, NP_849549; AtLOL1, NP_001117399; AtLOL2, BAB93279), *Brassica oleracea* (BoLSD1, AAL50981; BoLSD2, AAL50982), *Populus trichocarpa* (Pt-EEE85297; Pt-EEE97678) and *Zea mays* (ZmLOL1, ACG45100; Zm-CBC44879; Zm-ACR35025). AtLOL2 and Os-BAB93279 (named OsLOL2 in Xu and He 2007) contain two zf-LSD1 domains, whereas the others contain three zf-LSD1 domains

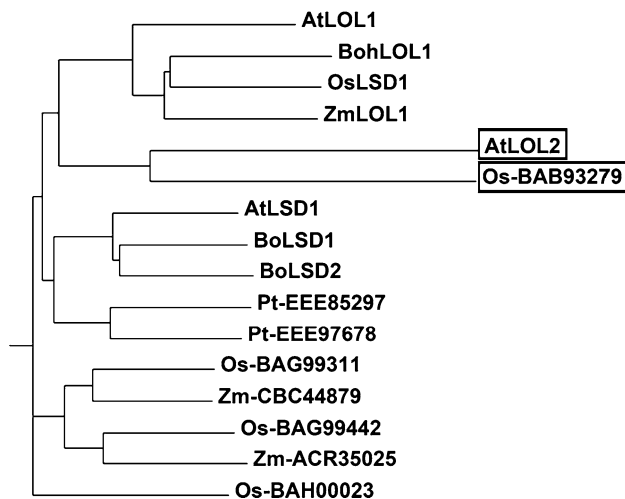


Fig. 2 Phylogenetic tree of LSD1 and LSD1-like proteins from different plants. The full-length amino acid sequences of LSD1 and LSD1-like proteins from *B. oldhamii* (BohLLO1, GenBank accession number ABW70167), *Oryza sativa* (OsLSD1, AAS13688; Os-BAG99442; Os-BAH00023; Os-BAB93279), *Arabidopsis thaliana* (AtLSD1, NP_849549; AtLOL1, NP_001117399; AtLOL2, BAB93279), *Brassica oleracea* (BoLSD1, AAL50981; BoLSD2, AAL50982), *Populus trichocarpa* (Pt-EEE85297; Pt-EEE97678) and *Zea mays* (ZmLOL1, ACG45100; Zm-CBC44879; Zm-ACR35025) were aligned using the CLUSTAL W program. A phylogenetic tree was generated using the program Phylodendron (version 0.8d, beta). The proteins that contain two zf-LSD1 domains are boxed

expression vector (pTrcHisA) were cultured under the same conditions for induction of the recombinant BohLLO1, and the proteins were purified by cobalt-based IMAC in parallel for use as a negative control in DNA-binding assays. Figure 7b shows the results of the filter-binding assay. The amount of ³²P-labeled bamboo genomic DNA fragments retained on nitrocellulose filters increased as the amount of recombinant BohLLO1 increased; this suggested that the protein directly interacted with the DNA. Southwestern analysis using ³²P-labeled bamboo genomic DNA fragments as a probe further confirmed the DNA-binding activity of the recombinant BohLLO1 (Fig. 7a, right panel).

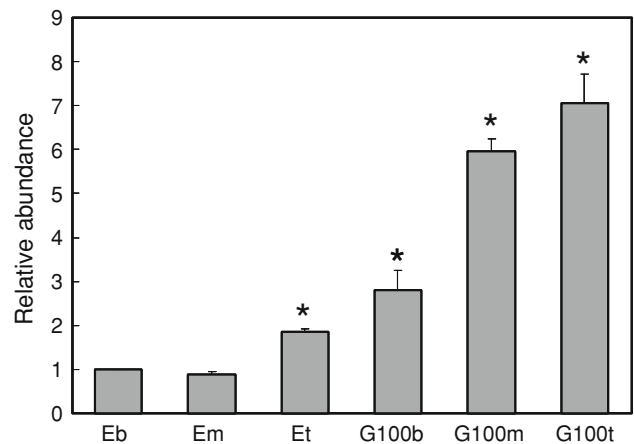


Fig. 3 Expression of *BohLLO1* in developing bamboo shoots. Total RNA was isolated from etiolated shoots (E) and 100-cm green shoots (G100). Each shoot was divided into three parts: the base (b), the middle (m) and the top (t). The levels of *BohLLO1* mRNA were analyzed by real-time RT-PCR. The *BohLLO1* mRNA levels in different samples were calculated relative to the base of the etiolated shoot (Eb), which was set to 1. The mean values \pm SD of three independent experiments are shown. The asterisks indicate significant differences ($P < 0.01$) between Eb and other samples

Subcellular localization of BohLLO1

The DNA-binding activity of BohLLO1 indicated that it was a nuclear protein, although no nuclear localization sequence was found in its deduced amino acid sequence. To investigate the localization of BohLLO1 in cells, BohLLO1 was translationally fused to the C terminus of the sGFP (Chiu et al. 1996) under the control of the CaMV 35S promoter, and the chimeric construct was transformed into the protoplasts of bamboo cells. The transient expression of the fusion proteins showed that sGFP::BohLLO1 proteins were predominately detected in the nuclei (Fig. 8a). Similar results were observed when the chimeric construct was transformed into onion epidermal cells for expression analysis (data not shown). For further confirmation, proteins from cytosolic and nuclear fractions were subjected to western analysis. As shown in Fig. 8b, BohLLO1 was detected in the nuclear fraction.

Discussion

The expression of *BohLLO1* is closely related to responses of biotic stress and the bamboo growth

In this study, we cloned a cDNA (*BohLLO1*) that encodes a protein containing three zf-LSD1 domains from bamboo. Because reverse genetic approaches have not yet been established for bamboo, it is difficult to directly assess the role of this gene. Nevertheless, at least two functions were suggested for BohLLO1 based on the results of the

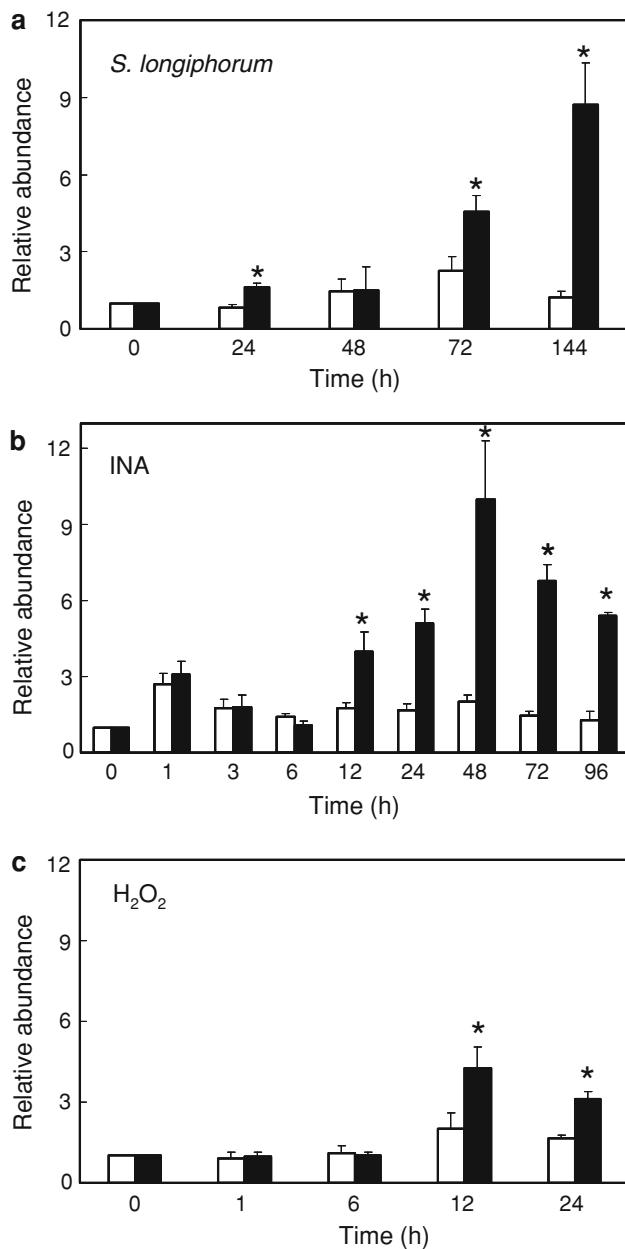


Fig. 4 Effects of pathogen infection, INA and hydrogen peroxide on *BohLOLI* expression in multiple shoots of bamboo cultured in vitro. Multiple shoots were infected with the bamboo pathogen *S. longiphorum* (a) or treated with 500 μ M INA (b) or 10 mM H₂O₂ (c) for the indicated time intervals (black column). Untreated shoot samples (white columns) were collected in parallel. The levels of *BohLOLI* mRNA in different samples were analyzed by real-time RT-PCR. The levels of *BohLOLI* mRNA in different samples were calculated relative to those in samples collected at the beginning of the treatment (0 h), which were set to 1. The mean values \pm SD of three independent experiments are shown. The asterisks indicate significant differences ($P < 0.01$) between untreated and treated samples at each time point

real-time RT-PCR. First, the increase in *BohLOLI* mRNA levels in response to *S. longiphorum* infection and INA treatment suggests that *BohLOLI* participates in disease

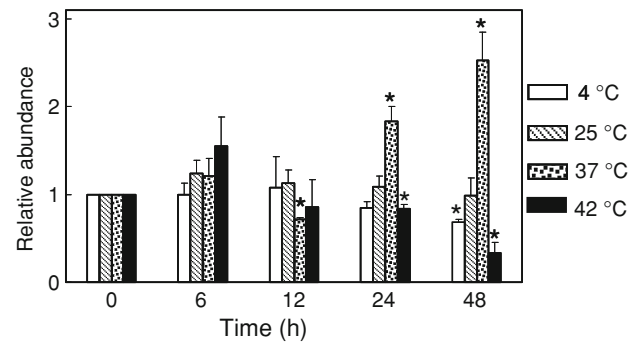


Fig. 5 Effects of temperature on *BohLOLI* expression in multiple shoots of bamboo cultured in vitro. The levels of *BohLOLI* mRNA in the multiple shoots cultured at various temperatures for different time intervals were analyzed by real-time RT-PCR. For each temperature utilized, the levels of *BohLOLI* mRNA in samples collected at different time points were calculated relative to the control (0 h), which was set to 1. The mean values \pm SD of three independent experiments are shown. The asterisks indicate significant differences ($P < 0.01$) between the control and a subsequent time point

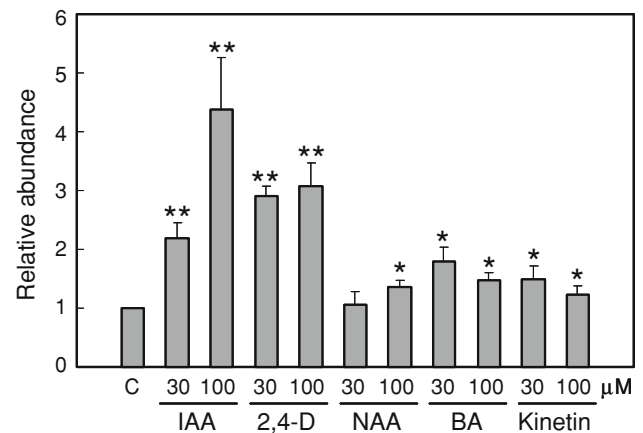


Fig. 6 Effects of auxins and cytokinins on *BohLOLI* expression in multiple shoots of bamboo cultured in vitro. Multiple shoots were incubated with 30 or 100 μ M auxins (IAA, 2,4-D or NAA) or cytokinins (BA or kinetin) for 12 h. The level of *BohLOLI* mRNA in each sample was analyzed by real-time RT-PCR. The level of *BohLOLI* mRNA in shoots without phytohormones added (control, C) was set to 1 to calculate the relative levels of *BohLOLI* mRNA in phytohormone-treated samples. The mean values \pm SD of three independent experiments are shown. The asterisks indicate significant differences ($*P < 0.05$, $**P < 0.01$) between the control and a treated sample

resistance and an SA-derived signaling pathway. Second, the significant up-regulation of *BohLOLI* expression in bamboo shoots with higher rates of culm elongation and in response to auxin treatments suggests a possible role of *BohLOLI* in growth. The positive effects of cytokinins, although not as marked as auxin treatments, also support this suggestion. In addition, the accumulation of *BohLOLI* mRNA increased more than twofold when the multiple shoots were treated with H₂O₂, indicating that H₂O₂ or an

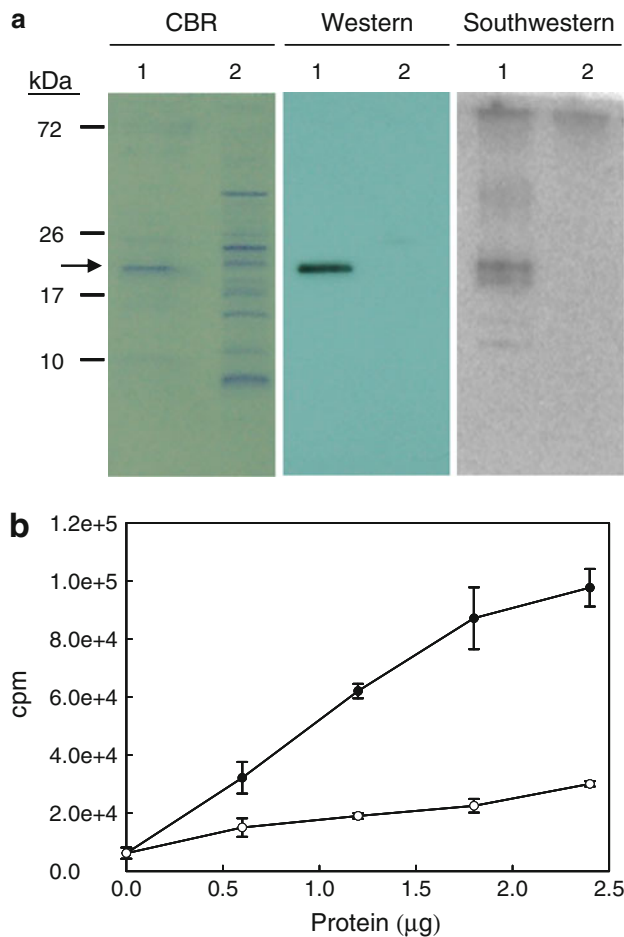


Fig. 7 DNA-binding assays of BohLOL1. **a** Recombinant BohLOL1 proteins that were expressed in *E. coli* TOP10 were purified by cobalt-based IMAC and separated on tricine SDS-PAGE. The proteins in the gel were stained with Coomassie blue (CBR, left panel) or transferred to a PVDF membrane and then immuno-detected with an anti-His antibody (middle panel) or hybridized with ³²P-labeled bamboo genomic DNA fragments (right panel). Lane 1, purified recombinant BohLOL1 proteins; lane 2, proteins purified from IPTG-induced *E. coli* harboring the expression vector pTrcHisA by cobalt-based IMAC as a negative control. The arrow marks the recombinant BohLOL1 protein band. **b** Purified recombinant BohLOL1 proteins (closed circle) and negative control proteins (open circle) were incubated with ³²P-labeled bamboo genomic DNA fragments and filtered through nitrocellulose membranes. The radioactivities of the DNA–protein complexes retained on the membranes were analyzed by a scintillation counter

H₂O₂-derived signal, which is usually produced under stress as well as during growth and development, is involved in BohLOL1 function. Moreover, the expression of *BohLOL1* was up-regulated when the in vitro cultured multiple shoots were incubated at 37°C, which is close to the general growth temperatures for the bamboo plants grown in the field. Under cold stress, at which the shooting and growth of bamboo culms does not usually occur, the

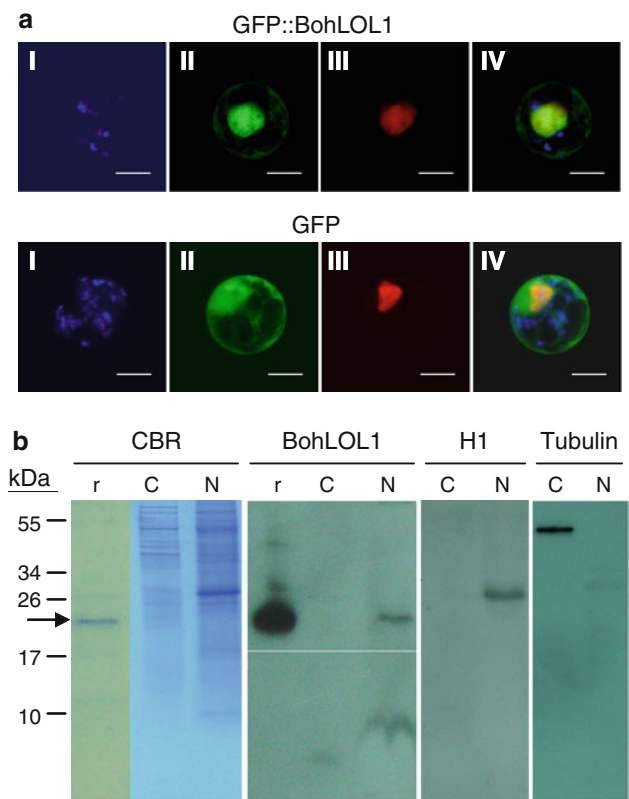


Fig. 8 Subcellular localization of the BohLOL1 protein. **a** Plasmids pHBT95sGFP-LOL1 (GFP::BohLOL1) or pHBT95sGFP (GFP) were co-transformed with a plasmid carrying *VirD2-NLS-mCherry* into bamboo protoplasts. Transformed bamboo protoplasts were visualized by laser scanning confocal microscopy: I autofluorescence; II fluorescence from sGFP; III fluorescence from mCherry, which shows the localization of the nucleus; and IV merged image. Bar = 10 µm. **b** Western analysis of BohLOL1 in different subcellular fractions of bamboo shoots. The cytosolic proteins and nuclear fractions were separated on tricine SDS-PAGE and then stained with Coomassie blue (CBR) or transferred to PVDF membranes. The membranes were immuno-detected with antibodies against BohLOL1, human histone H1 (H1) and sea urchin α -tubulin (Tubulin). Histone H1 and α -tubulin are nuclear and cytoplasmic marker proteins, respectively. r partially purified recombinant BohLOL1, C cytosolic fraction, and N nuclear fraction. Forty µg of proteins were loaded in each lane, and proteins in the nuclear fraction were enriched approximately 200-fold compared to the cytosolic fraction. The arrow marks the BohLOL1 protein band

transcript levels of *BohLOL1* decreased gradually after 24 h, though the levels of change were low. The down-regulation of *BohLOL1* expression was also observed after 48 h of heat-stress treatment. The expression patterns of *BohLOL1* indicate that it is not a constitutively expressed gene, and changes in its expression level might be connected to the plant's responses to different growth conditions.

The function of BohLOL1 in pathogen defense was anticipated because its orthologs (AtLOL1 and OsLSD1)

and its homolog (AtLSD1) are involved in the regulation of cell death in response to pathogen infection, though they do not play the same role; AtLSD1 and OsLSD1 are negative regulators of PCD, while AtLOL1 is a positive regulator (Epple et al. 2003; Wang et al. 2005). We are unable to resolve whether BohLOL1 is a regulator of PCD based on the results of this study. However, based on the observed expression patterns, we conclude that BohLOL1, unlike its ortholog OsLSD1, is not likely to be an exact functional homolog of AtLSD1. As described earlier, *BohLOL1* expression can be upregulated by pathogen infection and INA treatment and was differentially expressed in various tissues of developing bamboo shoots. Conversely, low-level, constitutive expression of *AtLSD1* in various tissues and in detached, senescent leaves of *Arabidopsis* has been observed (Dietrich et al. 1997; Coupe et al. 2004); furthermore, INA treatment was unable to affect its expression level (Dietrich et al. 1997). This difference in expression patterns suggests that AtLSD1 and BohLOL1 are not controlled in the same manner and may participate in different regulatory mechanisms.

The potential function of BohLOL1 in growth was unexpected, as the up-regulation of gene expression in growing tissues has not been reported for AtLSD1 or other proteins containing three zf-LSD1 domains. As with AtLSD1, AtLOL1 in *Arabidopsis* and OsLSD1 in rice have both been reported as constitutively expressed in various tissues (Epple et al. 2003; Wang et al. 2005). Although OsLSD1 in rice has been proposed to play a positive role in callus differentiation (Wang et al. 2005), this function was defined under conditions of overexpression of *OsLSD1* in transgenic rice lines. However, a parallel relationship was observed between the endogenous *BohLOL1* mRNA levels and the rate of bamboo elongation.

The involvement of proteins of the LSD1-like family in both plant growth and disease resistance has also been reported for rice OsLOL2 (GenBank accession number BAB93279 or AU031595) (Xu and He 2007). This protein contains two zf-LSD1 domains and the sequences outside these two conserved domains share no essential homology with the proteins containing three-zf-LSD1 domains (Fig. 1) (Xu and He 2007). The role of OsLOL2 in rice growth was defined by the dwarf phenotype of *OsLOL2* antisense transgenic lines (Xu and He 2007). It was proposed that the sequence outside of the two zf-LSD1 domains might signify its role in growth (Xu and He 2007). By adding our findings to the known roles of AtLSD1, AtLOL1, OsLSD1 and OsLOL2, we propose that the plant LSD1-like protein family may possess common functions in disease resistance and some members in this family may also have functions in plant growth; each member of this family has its specific role and may be involved in different regulatory pathways in different species.

The biochemical identity of BohLOL1 as a nuclear DNA-binding protein reveals that this protein performs its functions through protein-DNA interactions

AtLSD1, AtLOL1 and OsLSD1 have been proposed to function as transcription factors or protein scaffolds (Dietrich et al. 1997; Epple et al. 2003; Wang et al. 2005), but no direct evidence has been reported. The OsLSD1-GFP fusion protein was detected in the nucleus (Wang et al. 2005); however, AtLSD1 was found to be a cytoplasmic protein that keeps the *Arabidopsis* transcription factor AtbZIP10, a positive cell death regulator, outside the nucleus under pathogen infection (Kaminaka et al. 2006). In the present study, the results of subcellular localization studies and DNA-binding activity assays clearly demonstrate that BohLOL1 is a nuclear DNA-binding protein. The differences in their subcellular localization support the idea that BohLOL1 and AtLSD1 are involved in different regulatory mechanisms. In addition, the biochemical identity of BohLOL1 as a DNA-binding protein suggests that this protein performs its functions through protein-DNA interactions and also possibly through protein-protein interactions, and then affects the expression of target genes that participate in disease resistance and plant growth.

In summary, the data presented here suggest a possible role of BohLOL1 in plant growth and disease resistance; in addition, the data indicate that bamboo possesses unique mechanisms for controlling BohLOL1 levels to respond to physiological requirements and environmental changes. The up-regulation of *BohLOL1* expression that uniquely occurs in growing bamboo might be one of the critical factors contributing to the rapid growth that distinguishes this remarkable plant.

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