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Plant gene responses to frequency-specific sound signals

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Abstract We identified a set of sound-responsive genes in plants using a sound-treated subtractive library and demonstrated sound regulation through mRNA expression analyses. Under both light and dark conditions, sound up-regulated expression of *rbcS* and *ald*. These are also light-responsive genes and these results suggest that sound could represent an alternative to light as a gene regulator. *Ald* mRNA expression increased significantly with treatment at 125 and 250 Hz, whereas levels decreased significantly with treatment at 50 Hz, indicating a frequency-specific response. To investigate whether the *ald* promoter responds to sound, we generated transgenic rice plants harboring a chimeric gene comprising a fusion of the *ald* promoter and *GUS* reporter. In three independent transgenic lines treated with 50 or 250 Hz for 4 h, *GUS* mRNA expression was up-regulated at 250 Hz, but down-regulated at 50 Hz. Thus, the sound-responsive mRNA expression pattern observed for the *ald* promoter correlated closely with that of *ald*, suggesting that the 1,506 bp *ald* promoter is sound-responsive. Therefore, we propose that in transgenic plants, specific frequencies of sound treatment could be used to regulate the expression of any gene fused to the *ald* promoter.

Keywords Aldolase · Frequency-specific expression · Rice · Sound-induced genes

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Introduction

Like all living organisms, plants have highly complex sensory networks for monitoring their surroundings, and need to modify their growth and development to suit their environment. For example, plants exposed to a variety of mechanical perturbations, such as wind or touch, undergo physiological and developmental changes that enhance resistance to subsequent mechanical stress. Developmental changes in response to mechanostimulation are collectively known as thigmomorphogenesis (Braam et al. 1977; Johnson et al. 1998; Braam 2005). Both physiological and developmental changes occur in response to altered

environmental conditions and these results form rapid and dramatic fluctuations in plant gene expression. These molecular responses aid plants to acclimate or withstand stresses experienced under different environmental conditions.

The expression levels of several genes are known to change in response to external stimuli and for example, the touch (TCH) genes are induced by mechanostimulation (Braam et al. 1977; Braam 1992; Sistrunk et al. 1994; Braam 2005). In *Arabidopsis*, expression of TCH genes may be up-regulated 10- to 100-fold following not only mechanical stimuli, such as touch and wounding, but also darkness, temperature extremes and some growth-promoting hormones. The existence of distinct gene sets that respond to different stimuli suggests that specific receptors and signal transduction pathways are utilized for responses to alterations in environmental conditions. Mechanotransduction has been reported in animal cells and tissues and mechanical stresses have been shown to stimulate various signal transduction mechanisms (Bhagyalakshmi et al. 1992; Mizoguchi et al. 1996; Johnson et al. 1998). Bhagyalakshmi et al. (1992) reported an increase in inositol triphosphate and diacylglycerol levels when endothelial cells were subjected to blood flow-induced shear stress. Osteoblasts that were subjected to similar mechanical stresses exhibited activation of adenylate cyclase and increases cAMP levels (Reich et al. 1990). Additional shear-stress-induced genes include the gene encoding platelet-derived growth factor (PDGF), as well as *fos* and *jun* (Hsieh and Frangos 1991; Hsieh et al. 1993). There is also evidence that following wounding or other forms of mechanical stimulation, protein kinase levels increase rapidly in the mechanostimulus response pathway of plants (Mizoguchi et al. 1994, 1995, 1996; Bogre et al. 1996). Thus, mitogen-activated proteins such as Ca^{2+}-dependent protein kinase (Braam and David 1990; Knight et al. 1991; Braam et al. 1992; Sistruck et al. 1994) and ribosomal protein S6 kinase (Mizoguchi et al. 1995), might be involved in the mechanostimulus signal transduction pathway.

Sound is a disturbance (vibration) of energy in gas, liquid or solid and there are some reports that sound vibrations can stimulate seed germination and plant growth (Weinberger and Burton 1981; Takahashi et al. 1992; Creath and Schwartz 2004). Takahashi et al. (1992) reported that treatment of germinating rice or cucumber seeds with a 50 Hz vibration for 72 h resulted in stimulation of the hypocotyl elongation rate. Similar results have been reported in *Arabidopsis* (Johnson et al. 1998). Through biochemical analyses, Bouch et al. (2004) demonstrated that increased levels of indole acetic acid (IAA) and decreased levels of abscisic acid (ABA) correlated with sound wave stimuli. Following treatment of *Chrysanthemum* with a specific frequency (1.4 kHz, 0.095 kdB, 10 days) the levels of IAA and ABA were significantly higher and lower, respectively. The researchers determined that greater relative levels of IAA/ABA favored development of the callus, as well as differentiation of the mature callus. They concluded that sound waves contributed to the regulation of endogenous hormone levels, as well as to the control of callus growth.

Previous research into the effects of music and/or noise on plants has been controversial (Weinberger and Das 1972; Weinberger and Measures 1978; Galston and Slayman 1979) and has proved difficult to replicate because the precise experimental conditions were not specified. Thus, despite the ecophysiological importance of sound wave acclimation, little is known about the molecular–physiological mechanisms underlying such a response. In this study, we investigated whether sound could alter gene expression in rice plants. Using both complex musical sounds and single frequencies, we isolated several sound-induced genes. mRNA analyses demonstrated frequency-specific regulation of expression of the gene encoding the cytoplasmic protein aldolase (*ald*).

Materials and methods

Plant materials and sound treatment

Rice plants (*Oryza sativa* L. Donggin) were cultivated in a growth chamber under continuous light at 28°C and 65–75% relative humidity (RH). After two weeks, the plants were subjected to two different kinds of sound treatment, classical music and single frequency. We chose 14 different classical music pieces including: Piano Sonata No. 14 in C Sharp Minor, Op.27 No.2 ‘‘Moonlight” 1st Mov. (Beethoven); Clair De Lune from Suite Bergamasque (Debussy); Wedding March from A Midsummer Night’s Dream (Mendelssohn); Hungarian Dance

No. 5 (Brahms); Symphony No. 104 in a D Major ''London'' 3rd Mov. Menuetto:-Allegro (Haydn); Winter from The Four Seasons 1st Mov. Allegro Non Molto (Vivaldi); The Arrival of the of Queen Sheba from Solomon (Handel); Piano Sonata No. 8 in C Minor, Op.13 ''Pathetique'' 1st Mov. (Beethoven); Variation 25 from the Goldberg Variations BWM-988 (J. S. Bach); Can-Can from Orpheus in the Underworld (Offenbach); Piano Quartet, K-493 2nd Mov. Larghetto (Mozart), Clarinet Concerto in a Major K-622 2nd Mov. Adagio (Mozart); Brandenburg Concerto No. 5 in D Major, BWV 1050 1st Mov. and Allegro (J. S. Bach); and Ave Maria (Bruckner).

The single frequency signal was generated using the software provided by the Center for Noise and Vibration Control (NOVIC) of the Advanced Institute of Science Technology (KAIST) in Korea. During the sound treatments, the speaker and plants were placed on different shelves to prevent transfer of vibrations. The speaker volume was set between 65 and 70 dB and to prevent any effects from extraneous noise, a noiseless growth chamber with internal sound levels of about 40 dB was custom-made (Korea Scientific Technique Industry Co., Korea). Generally, the sound level in a commercial growth chamber is about 80 dB.

Construction of a sound-induced subtractive cDNA library and isolation of sound-induced genes

To isolate sound-induced genes, we constructed a sound-induced subtractive cDNA library using mRNA isolated from the leaves of two-week-old rice plants treated with classical music for 4 h. Total RNA was isolated from the leaves of sound-treated and untreated control plants using Trizol Reagent (Gibco-BRL). Poly(A)$^+$ RNA was purified using biotinylated Oligo(dT)$_{25}$. Sound-treated cDNA was synthesized using the SMART polymerase chain reaction (PCR) cDNA Synthesis kit (Clontech, USA). Subsequent selective subtraction was performed using Streptavidin MagneSphere Magnetic separation products (Promega, USA), according to the manufacturer's instructions. Double-stranded cDNA was synthesized from subtracted mRNA using the SMART PCR cDNA Synthesis kit and after the second round of amplification, PCR products were cloned directly into pGEM-T Easy (Promega, USA), then transformed into *Escherichia coli* XL1-Blue. DNA was isolated from white clones and dot-blotted onto duplicate Hybond-N membranes (Amersham Pharmacia Biotech, London, UK). In order to identify sound-responsive genes, these membranes were hybridized with either [α-^{32}P] dCTP-labeled total cDNA from untreated or sound-treated plants.

Northern blot and RT-PCR analyses

In order to analyze mRNA expression of sound-induced genes selected from a subtractive cDNA library, we performed Northern blot analysis and quantitative reverse transcription (RT)-PCR. For Northern blot analysis, total RNA (20 μg) from the leaves of sound-treated and untreated control plants were separated by agarose gel electrophoresis and transferred to a membrane. Hybridization was performed at 65°C for 16 h, as described by Sambrook et al. (1989). cDNA probes were generated for using the genes encoding a putative fructose 1,6-bisphosphate aldolase (*ald*), ribulose 1,5-bisphosphate carboxylase (Rubisco) small subunit (*rbcS*), a DNA-J-like protein and calreticulin. Analyses of the *GUS* and *ald* gene expression in P*ald::GUS* transgenic rice plants were performed by RT-PCR using the *GUS*-specific primer set (sense: 5′-ATGTTACGTCCTGTAGAA ACC-3′ and antisense: 5′-CTAAAGAGAGGTTA AAGCCGA-3′) and an *ald*-specific primer set (sense: 5′-AGAAAGCTGGGTTGCTTCCGGTTTCTCTGA AGG-3′ and antisense: 5′-AAAAAGCAGGCTTG-CATCACAGACACATTTGTT-3′). For the control of PCR amplification, *actin* gene was used. The sequences of *actin*-specific sense and antisense primers are 5′-ATGGCTGACGCCGAGGATATCCAG-3′ and 5′-TTAGAAGCATTTCCTGTGCACAAT-3′, respectively. For the reverse transcription reaction, the first strand synthesis was performed in an 80 μl reaction mixture containing 5× buffer (Promega, USA), 0.25 mM each dNTP, 2 μg *GUS*-specific primers, 200 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, USA), and 8 μl DNase-treated RNA preheated at 72°C for 2 min. The reaction was incubated at 37°C for 1 h. PCR amplification was performed in a thermal cycler PTC-200 (MJ Research, USA) using the following cycling parameters; 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for

2 min and a final extension of 72°C for10 min. The PCR conditions for amplification of the *actin* and *ald* gene were identical to that of *GUS* cDNA synthesis except the annealing temperature. The annealing temperatures were 55°C and 68°C for the *ald* gene and *actin* gene, respectively. RT-PCR products were separated by electrophoresis on 0.8% agarose gel.

Construction of plasmids containing *ald* promoter-GUS transcriptional fusions

The *ald* promoter fragment was obtained by PCR amplification of wild-type rice (*O. sativa* L. Donggin) and comprised the region from −1 to −1200 bp, relative to the transcriptional start. Following subcloning into pGEM-T Easy vector and sequencing, PCR amplification was performed using an *ald* promoter-specific primer set, in which the *attB1* and *attB2* sites were added to the 5′ ends of the forward and reverse primers, respectively. This PCR fragment was introduced by recombination into the entry vector pDONR-201, using a BP clonase enzyme mixture (Invitrogen, USA). The recombination reactions (BP reactions) were then used for heat shock transformation of competent *E. coli* XL-1 Blue and kanamycin-resistant colonies were selected. Plasmid DNA was isolated and used for a second reaction with the LR clonase enzyme mixture (Invitrogen, USA), in which the fragment was recombined into the destination vector pBGWFS7, containing the *β*-glucuronidase reporter (*GUS*) and *bar* selection. *E. coli* was transformed with the LR reaction and the recombinant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 using freeze–thaw method (Hofgen and Willmitzer 1988).

Agrobacterium-mediated transformation and regeneration of transgenic rice plants

Plasmid constructs were introduced into Donggin rice cells using *Agrobacterium*-mediated gene transfer, according to the method of Hiei et al. (1994), with some modifications. Mature seeds were dehusked, sterilized with 70% (v/v) ethanol for 2–3 min, transferred into 50% (v/v) commercial bleach solution (Clorox) for 40 min, and then rinsed with sterile water and plated on Murashige & Skoog (MS) media (Sigma, USA) supplemented with 4.41 g l^{-1} MS mixture; 30 g l^{-1} sucrose; 3.0 g l^{-1} phytagel, pH 5.8 and grown for 3–4 weeks at 25°C in the dark. Approximately 100–150 embryogenic calli were immersed and shaken gently for 20 min in a suspension of *A. tumefaciens* (harboring pBGWFS7-P*ald*), then transferred onto 2N6-AS media (Duchefa, Netherlands, 4.4 g l^{-1} N6 vitamins mixture; 25 mg l^{-1} Fe-EDTA; 300 mg l^{-1} Casein enzymatic hydrolysae; 500 mg l^{-1} proline; 500 mg l^{-1} glutamine; 30 g l^{-1} sucrose; 2 mg l^{-1} 2,4-dichlorophenoxy acetic acid; 2.5 g l^{-1} phytogel; glucose 10 g l^{-1} ; 200 μM acetosyringone, pH 5.8) for 3 days of co-cultivation at 25°C in the dark. The calli were then rinsed with sterile water containing 250 mg l^{-1} cefotaxime and blotted onto filter paper.

These calli were placed immediately onto 2N6-CP selection media (4.4 g l^{-1} N6 vitamins mixture; 25 mg l^{-1} Fe-EDTA; 300 mg l^{-1} casein enzymatic hydrolysae; 500 mg l^{-1} praline; 30 g l^{-1} sucrose; 500 mg l^{-1} glutamine; 2 mg l^{-1} 2,4-dichlorophenoxy acetic acid; 2.5 g l^{-1} phytogel; 200 μM acetosyringone; 250 mg l^{-1} cefotaxime; 3 mg l^{-1} phosphinotricin; pH 5.8) and cultured at 25°C for 2–3 weeks in the dark. They were sub-cultured twice on fresh 2N6-CP media and grown for 2 weeks each time. Actively growing calli were excised and cultured on MSR-CP media (4.41 g l^{-1} MS mixture; 0.5 mg l^{-1} NAA; 2 mg l^{-1} kinetin; 30 g l^{-1} sucrose; 4 g l^{-1} phytogel; 250 g l^{-1} cefotaxime; 3 mg l^{-1} phosphinotricin) at 25°C for 2 weeks in the dark. Regenerated plantlets were acclimatized hydroponically in Yoshida nutrient solution (1976) for 10 days and putative transformants were transferred into soil in pots, where they were tested for Basta herbicide resistance.

Selection of transgenic rice plants using PCR analysis

Leaves from un-transformed control plants and 50 transgenic rice plants were frozen with liquid nitrogen and ground using a mortar pestle. Total rice genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide method (Woodhead et al. 1998) and a fragment containing *bar* was amplified by PCR using gene-specific primers (sense: 5′-CCGTACCGAGCGCAGGAACC-3′ and antisense: 5′-GGCAGCCCGATGACAGCGACCAC-3′). PCR reactions were performed using the following conditions: denaturation at 94°C for 5 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min and

72°C for 2 min, followed by a final extension at 72°C for 10 min. PCR products were separated by 0.7% agarose gel electrophoresis.

Results and discussion

Screening a subtractive library for sound-induced genes

A subtractive cDNA library was constructed from sound-treated rice plants. In order to confirm the efficiency of the subtractive process, cDNA probes were made from the mRNA isolated from untreated and sound-treated rice plants, then hybridized to 100 randomly-selected recombinant clones. Many of the clones exhibited differences in the intensity of hybridization (Fig. 1). Positive signals were observed for more than 20 clones that hybridized to the tester cDNA probe and the four clones that gave the strongest signals were selected for further analyses. Results from sequencing and BLAST searches of the GenBank/EMBL database indicated a high level of homology to well-known genes encoding metabolically-important proteins such as the Rubisco small subunit (*rbcS*), fructose 1,6-bisphosphate aldolase (*ald*), a DNA-J-like protein and calreticulin.

So far a large number of mechano-stimulus inducible genes have been isolated and characterized (Braam 2005). However, most of these genes are touch responsive. Although there are many reports about sound responsiveness at the physiological level, not many investigations have been performed regarding sound responsiveness at the gene level. As far as we know this is the first report of characterization of plant sound responsive genes.

Confirmation of gene induction by sound signals

To confirm whether the genes selected exhibited sound-induced expression, mRNA levels were analyzed using Northern blots and/or RT-PCR analyses. Results demonstrated that the mRNA expression of all four genes was induced significantly by sound treatment (Fig. 2). As light-induced expression has been reported for both *ald* and *rbcS*, we examined the expression pattern of these genes in rice plants grown under light or dark conditions for 10 days, and treated with classical music for 4 h. Sound induced *rbcS* and *ald* expression under both light and dark conditions (Fig. 3). Interestingly, these genes exhibited a similar level of expression with sound treatment in darkness as with light treatment alone. This result suggests that as a gene regulator, sound may be an alternative to light (Fig. 3).

It was shown that *Arabidopsis* touch inducible TCH gene was also induced by spraying not only with gibberellins but also with a variety of hormones such as auxin, cytokinin and abscisic acid (Braam and Davis 1990). Touch and sound stresses are basically very similar externally applied mechanical stresses. Although none of the known touch inducible genes has been isolated by the methods we have performed, further investigation on how our sound inducible genes will respond to other stresses such as touch and hormones will give an insight on the relationship between various mechanical responses.

Gene responses to different sound frequencies

As expression may be induced by different frequencies of sound, we analyzed the response of *ald* to various frequencies produced by a single

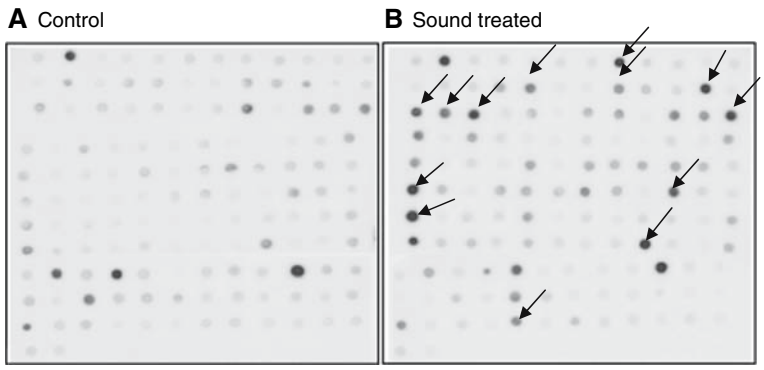

Fig. 1 Second screening of sound-induced rice genes isolated by reverse Northern blot analysis using un-subtracted total cDNA as probes. Duplicate membranes were hybridized with radiolabeled probes prepared from untreated (**A**), and treated cDNA (**B**). Differentially expressed clones are marked with arrows

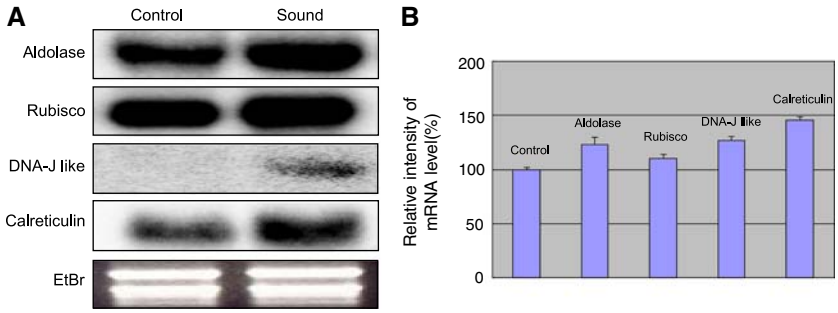

Fig. 2 Northern blot and RT-PCR analyses comparing four genes that exhibited differential expression using the driver and tester probes. Total RNA was isolated from rice plants treated with classical music for 4 h. Northern blot analyses were performed using radiolabeled cDNA probes for genes encoding aldolase, rubisco, and a DNA-J-like protein (**A**). cDNA encoding calreticulin was generated using a specific anti-sense primer, followed by PCR amplification with gene-specific sense and anti-sense primers (**B**). Equal loading of RNA was estimated by ethidium bromide staining of rRNA

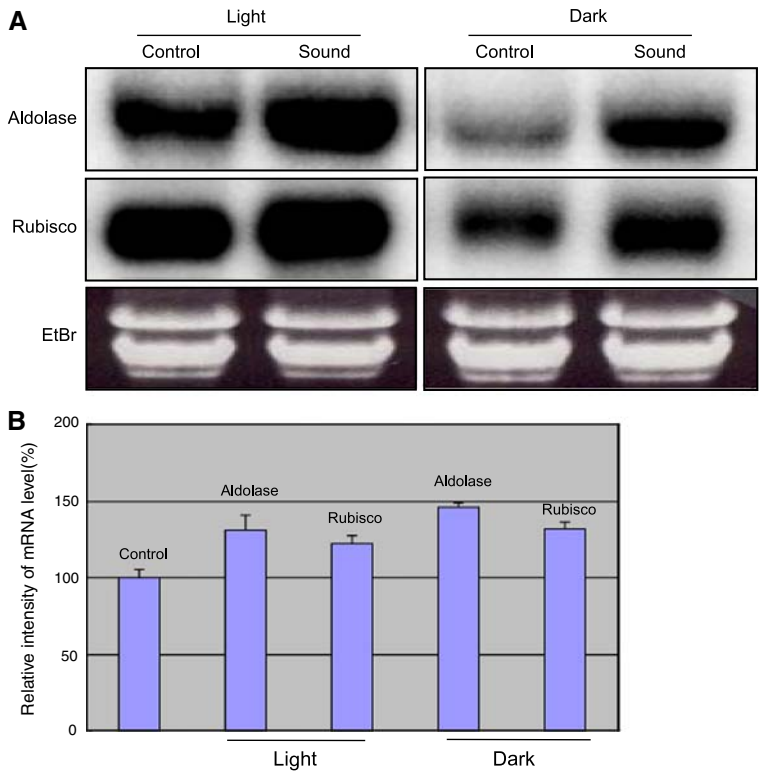

Fig. 3 Effect of sound treatments on light-treated *ald* and *rbcC* expression. mRNA expression levels were determined for rice plants grown under continuous light conditions (**A**) or transferred from light to continuous darkness for 10 day (**B**). Each lane was loaded with 15 μg total RNA extracted from rice plants subjected to sound treatment for 4 h. Blots were probed with (α-^{32}P) dCTP-labeled *ald* and *rbsC* cDNA. Equal loading was confirmed by ethidium bromide staining of total RNA

frequency sound generator in a noiseless growth chamber (less than 40 dB of noise). Two-week-old rice plants were subjected to various single frequencies for 4 h. There was a significant increase in *ald* mRNA levels at 125, 250 Hz or 1 kHz, but a significant decrease at 50 Hz (Fig. 4). In the 50 Hz treatment, mRNA expression of *ald* decreased significantly after 4 h, and became very weak after 8 h (Fig. 5). These results revealed that genes respond to sound in a frequency-specific manner. Temporal analysis of the 250 Hz treatment indicated that *ald* mRNA expression increased after

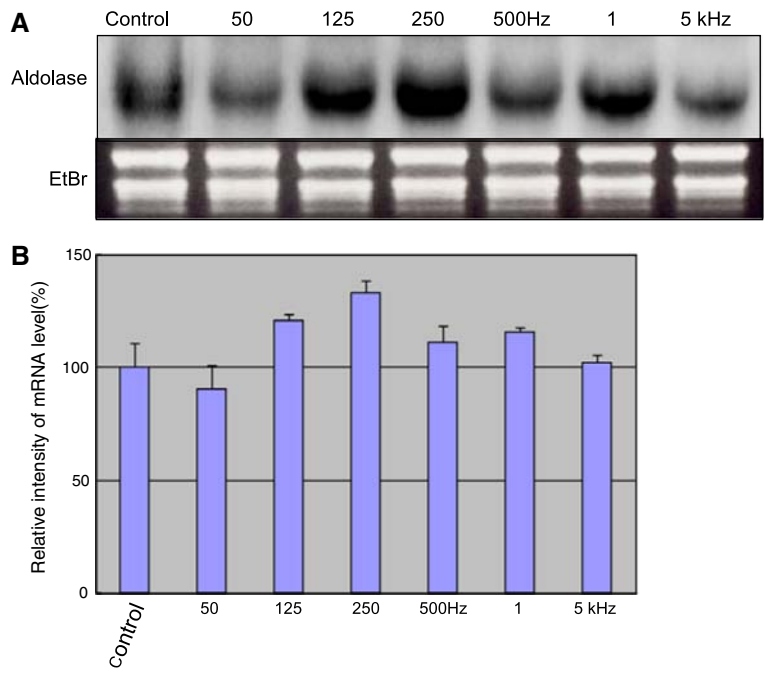

Fig. 4 Northern blot analysis of *ald* expression in rice plants treated for 4 h with the frequencies indicated. Blots were probed with (α-^{32}P) dCTP-labeled *ald* cDNA. Equal loading was confirmed by ethidium bromide staining of total RNA

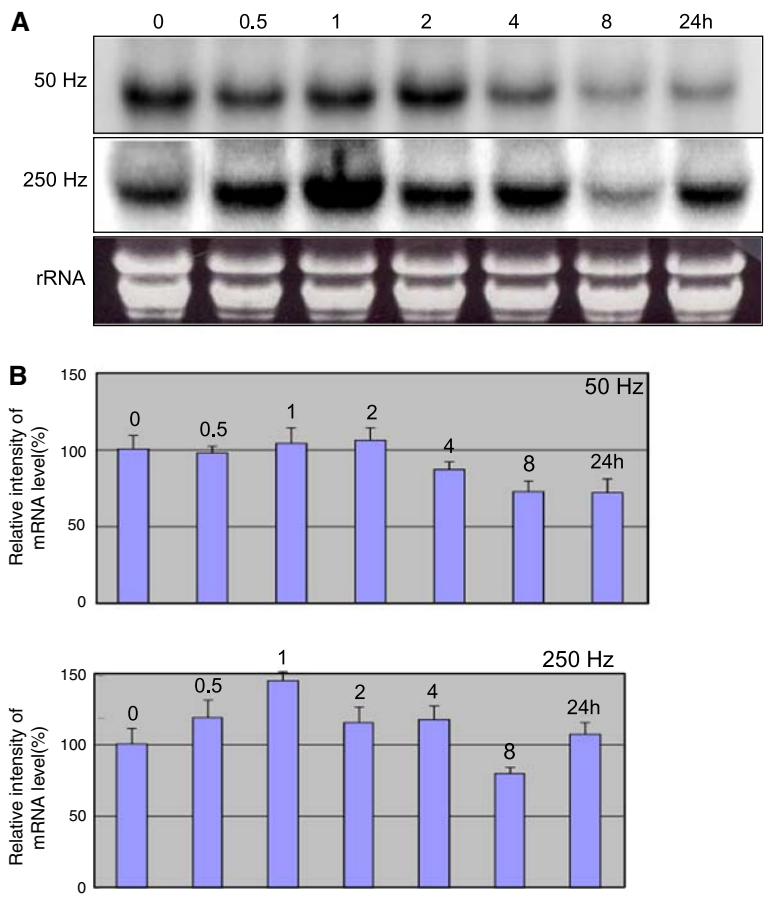

Fig. 5 Temporal analysis of specific frequency-induced *ald* mRNA expression in rice plants. At the times indicated, RNA was extracted from untreated control, and 50 and 250 Hz-treated plants. Blots were probed with (α-^{32}P) dCTP-labeled *ald* cDNA. Equal loading was confirmed by ethidium bromide staining of total RNA

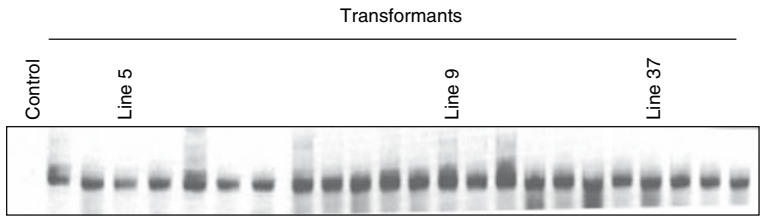

Fig. 6 RT-PCR analysis of *GUS* expression in P*ald* transgenic rice plants. RT-PCR analysis of *GUS* expression was performed using total RNA (1.5 μg) isolated from independent transgenic and wild-type rice plants

30 min, reached a maximum after 1 h, and then decreased slightly thereafter.

Light-responsive expression of the *ald* promoter in transgenic rice plants

In order to determine whether or not the *ald* promoter responds to light and sound signals, we generated transgenic rice plants harboring a chimeric gene comprising a 1,200 bp *ald* promoter fragment fused to the *E. coli GUS* reporter. We performed RT-PCR using *GUS*-specific primers to identify transgenic rice plants expressing *GUS* (Fig. 6). Three independent lines that expressed *GUS* at low, medium and high level, were used to investigate the response of the *ald* promoter to sound signals. Initially, to determine whether the *ald* promoter is light-regulated, two isogenic plants from each transgenic line were grown in the dark for 5 days, and then transferred to continuous white light for 24 h. RT-PCR was used to determine the *GUS* expression levels in RNA isolated from dark- and light-treated leaf tissues. All three transgenic lines showed high levels of *GUS* induction under the light conditions (Fig. 7), correlating closely to the expression pattern observed for *ald* (Fig. 3). Thus, the reporter expression pattern demonstrated that the 1,506 bp *ald* promoter is light-responsive in transgenic rice plants.

Sound-responsive expression of the *ald* promoter in transgenic rice plants

In order to determine whether the 1506 bp *ald* promoter is sound-responsive, we treated three transgenic lines that harbored the *ald* promoter-*GUS* reporter fusion, with 50 or 250 Hz for 4 h. RNA isolated from sound-treated leaf tissues was

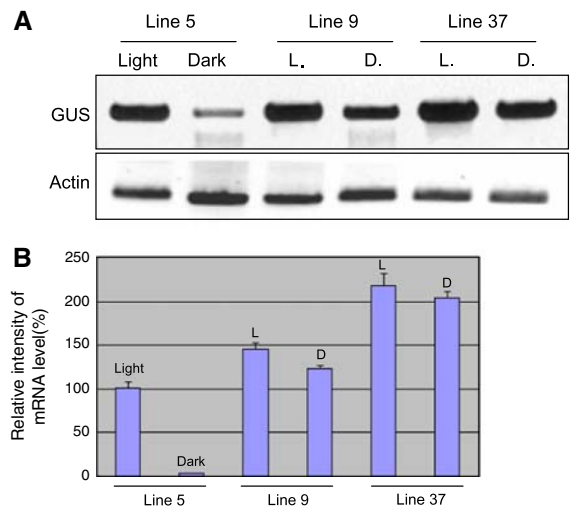

Fig. 7 RT-PCR analyses of *GUS* mRNA expression in P*ald* transgenic rice plants grown under dark and light conditions. Three transgenic rice plants (Lines 5, 9 and 37) were grown in darkness for 5 days, then allowed to recover under white light for 24 h. Total RNA was isolated from the leaves of dark-treated and light-recovered transgenic rice plants, then used for RT-PCR analysis of *GUS* expression. *Actin* cDNA was used as an internal PCR control

analyzed using RT-PCR to determine *GUS* mRNA expression levels. In all three transgenic lines, *GUS* expression was up-regulated at 250 Hz, but down-regulated at 50 Hz (Fig. 8). Thus, the reporter expression pattern demonstrated that the 1,506 bp *ald* promoter is responsive to specific frequencies and these results correlate closely to the expression pattern observed for *ald* (Fig. 5). In conclusion, we propose that in transgenic plants, specific frequencies of sound treatment could be used to regulate the expression of any gene linked to the *ald* promoter.

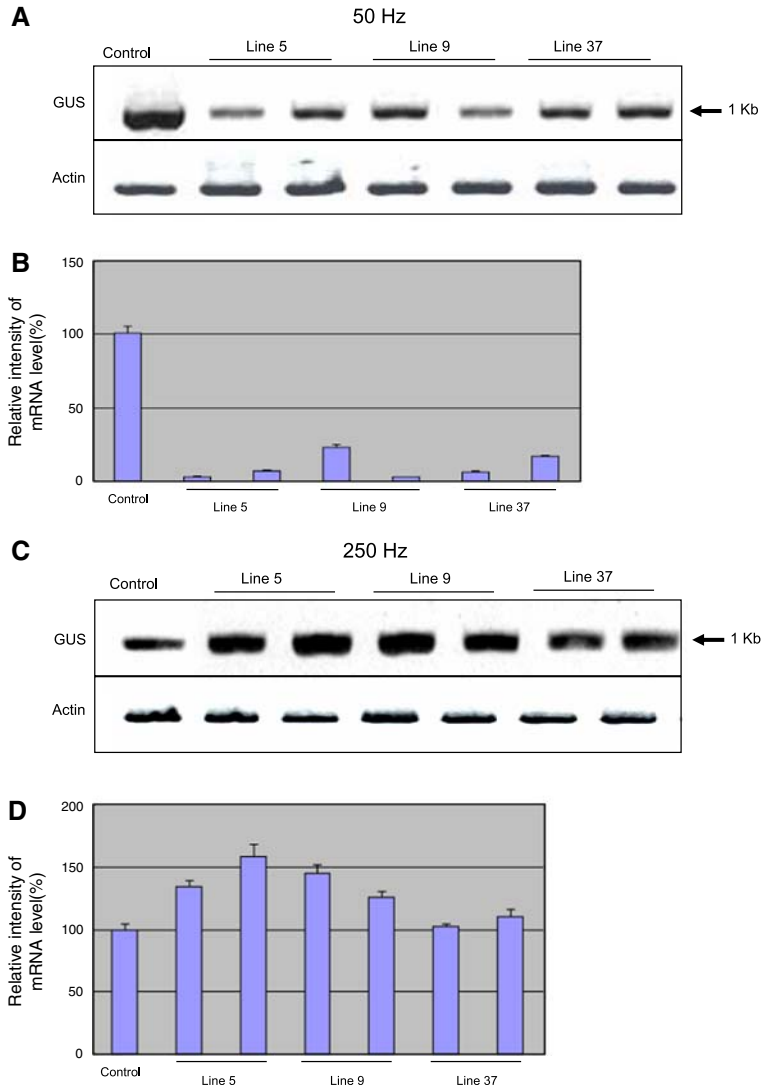

Fig. 8 Sound responsive expression of GUS gene in P*ald::GUS* transgenic rice plants. GUS gene expression was analyzed by RT-PCR amplification. Total RNA was prepared from independent transgenic rice plants treated with 50 Hz (**A**) and 250 Hz (**B**) signals for 4 h, and from untreated rice plants (control). Following reverse transcription, first-stand cDNA was used for PCR amplification with *GUS*-specific primers. The rice actin gene was used as control. PCR products were separated by agarose gel electrophoresis. The 1 kb fragment representing the *GUS* transcript is indicated by the arrows on the right. *Actin* cDNA was used as an internal PCR control

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