A novel zinc-binding alcohol dehydrogenase 2 from *Arachis diogoi*, expressed in resistance responses against late leaf spot pathogen, induces cell death when transexpressed in tobacco

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A novel zinc-binding alcohol dehydrogenase 2 (AdZADH2) was significantly upregulated in a wild peanut, *Arachis diogoi* treated with conidia of late leaf spot (LLS) pathogen, *Phaeoisariopsis personata*. This upregulation was not observed in a comparative analysis of cultivated peanut, which is highly susceptible to LLS. This zinc-binding alcohol dehydrogenase possessed a Rossmann fold containing NADB domain in addition to the MDR domain present in all previously characterized plant ADH genes/proteins. Transient over-expression of AdZADH2 under an estradiol inducible promoter (XVE) resulted in hypersensitive response (HR)-like cell death in tobacco leaf. However, the same level of cell death was not observed when the domains were transiently expressed individually. Cell death observed in tobacco was associated with overexpression of cell death related proteins, antioxidative enzymes such as SOD, CAT and APX and pathogenesis-related (PR) proteins. In *A. diogoi*, AdZADH2 expression was significantly upregulated in response to the plant signaling hormones salicylic acid, methyl jasmonate, and sodium nitroprusside.

Zinc-binding alcohol dehydrogenases are NAD(P)-dependent oxidoreductases involved in hydride ion transfer from alcohols to NAD$^+$ catalyzing reversible oxidation of alcohols to aldehydes or ketones. They are found in all plants, playing important roles in plant growth, pollen, and seedling development and fruit ripening [1,2]. They are well-studied members of the medium-length dehydrogenase/reductase (MDR) protein superfamily [3].

Expression of zinc-binding alcohol dehydrogenase was induced in Cyanobacterium PCC 6803 upon exposure to different environmental stresses [4] and in citrus upon interaction with *Citrus leprosis* virus [5]. Cinnamyl alcohol dehydrogenase (CAD), a zinc-binding MDR enzyme, is involved in lignin biosynthesis exhibiting defense-related activity and strengthening of cell walls [6]. A zinc-binding alcohol dehydrogenase is induced in Oomycete plant pathogenic fungus, *Phytophthora infestans* during the infection process [7]. Recently, another member of the MDR protein superfamily, an alcohol dehydrogenase gene of the fungus *Metarhizium anisopliae*, was found to be expressed during insect colonization and invasion [8].

In maize, *ADH1* transcripts increase significantly under anaerobic conditions [9]. Chung and Ferl [10] demonstrated that transgenic *Arabidopsis* plants

Abbreviations
ADH, alcohol dehydrogenase; AdZADH2, *Arachis diogoi*-zinc binding alcohol dehydrogenase 2; APX, ascorbate peroxidase; CAT, catalase; HIN1, harpin-induced1 gene; HMG-CoA R, 3-hydroxy-3-methyl-glutaryl-CoA reductase; HSR203J, hypersensitive response related 203j; LLS, late leaf spot; MDR, medium-length dehydrogenase/reductase; PR, pathogenesis related; RACE, rapid amplification of cDNA ends; SNP, sodium nitroprusside; SOD, superoxide dismutase; XVE, estradiol inducible promoter.
expressing ADH promoter–Gus fusions exhibited high Gus expression when grown under conditions simulating anoxia. When these plants are grown in conditions that mimic normal soil growth, the Gus expression was low, showing the relative levels of ADH under anoxia and normal soil conditions. In line with these observations, Ismond et al. [11] showed that the Arabidopsis-ADH1-null mutants exhibited decreased hypoxic survival. Dolferus et al. [12,13] studied the expression of the ADH gene in Arabidopsis and showed that the gene is induced by dehydration, cold and hypoxia and other environmental stresses. An alcohol dehydrogenase of Lotus japonicus (LjADH1) conferred tolerance to H2O2 induced stress in E. coli and yeast cells along with tolerance to heavy metal salts in the latter [14].

Myint et al. [15] analyzed the expression of alcohol dehydrogenase genes in Arabidopsis under polyethylene glycol mediated stress and observed upregulation of ADH1 and other ADH genes in the treatments indicating that the operation of drought tolerance probably involved ethanolic fermentation.

Several wild relatives of peanut including Arachis diogoi are highly resistant to many foliar diseases and biotic stresses [16]. In an earlier investigation, we have identified a Zinc-binding alcohol dehydrogenase 2 (AdZADH2) gene in Arachis diogoi in its interaction with Phaeoisariopsis personata that causes the serious late leaf spot disease in the cultivated peanut, Arachis hypogaea [17]. In this communication, we have focused on the cloning of the novel AdZADH2 and its involvement in HR-mediated cell death in tobacco.

Materials and methods

5’/3’ RACE-PCR, isolation of full length cDNA and transient conditional overexpression of AdZADH2

Rapid amplification of cDNA ends (RACE) was performed to clone the full length cDNA of AdZADH2 by using SMART™ RACE cDNA Amplification kit (Clontech, California, USA) using gene specific primers designed from the sequence of a partial fragment identified in an earlier study using cDNA-AFLP [17]. The ORF was amplified with primers AdZADH2-Apal-F and AdZADH2-SpeI-R containing Apal and SpeI restriction enzymes, respectively, by using Phusion™ DNA polymerase (Finzymes, Loughborough, UK, NEB, Table S1), cloned in pTZ57R/T and sequenced. The open reading frame (ORF) of the AdZADH2 with flanking Apal and SpeI sites was digested with appropriate restriction enzymes and cloned into pER8 vector (Gifted by N.-H. Chua, Rockefeller University, USA) to obtain the recombinant pER8:AdZADH2. We further cloned both the domains separately in pER8 vector as NADB Rossmann domain D1 (pER8:AdZADH2-D1) and MDR domain D2 (pER8:AdZADH2-D2) by using specific primers (AdZADH2-Apal-F and AdZADH2-D1-SpeI-R for D1 and AdZADH2-D2-Apal-F and AdZADH2-SpeI-R for D2) to obtain recombinant vectors pER8:AdZADH2-D1 and pER8:AdZADH2-D2 respectively (Fig. S5). Primer sequences used in this study are listed in Table S1.

Agro-infiltration, chemical treatment, and cell death assessment

The vectors were mobilized into Agrobacterium strain LBA4404 using freeze thaw method. Agrobacterium strains harboring different binary vector constructs were grown in Luria Broth in the presence of respective antibiotics, pelleted, resuspended and infiltrated as described earlier [18–20]. After the observation of cell death phenotype, the samples were collected, quick-frozen in liquid nitrogen and stored at −80 °C for further analysis.

Cell death quantification and ion leakage experiment

The extent of cell death was quantified by Evans Blue staining following Baker and Mock [21] method. For electrolyte leakage analysis, leaf discs (8 mm diameter) were cut off from agro-infiltrated regions after 48 h post infiltration using a cork borer and transferred to distilled water containing 30 μM 17β-estradiol in Petri dishes under bright light with 14/10 h photoperiod [17,22,23]. Electrolyte leakage values were determined by using an electro-conductivity meter (Digisun Electronics, Hyderabad, India) at different time intervals and data were plotted from three biological replicates.

Quantitative RT-PCR analysis post estradiol treatment

Leaves were infiltrated with agrobacterium strain LBA4404 harboring pER8, pER8:AdZADH2, pER8:AdZADH2-D1, and pER8:AdZADH2-D2 vectors, respectively. Total RNA was isolated from leaf samples with and without estradiol treatment collected at 0, 24, and 48 h time points. First strand cDNA was synthesized and used in the analysis of the transcript levels for several defense-associated genes along with HR marker genes using gene specific primers (Table S2). Actin was used as internal control for calculating relative gene expression. Relative fold change in RNA expression was estimated using ΔΔCt method [24].

Hormonal and SNP treatments

Twigs from the field-grown plants were cut and kept in a tray for 2 weeks on moist cotton saturated with sterile
Novel AdZADH2 of *Arachis diogoi* induces cell death

D. Kumar et al.

distilled water at the base and the tray was covered with a polythene sheet to maintain humidity for adventitious root formation and recovery. For various chemical treatments, the rooted twigs were kept in the corresponding solution. The treatments given were 100 μM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA), 250 μM ethephon, 100 μM sodium nitroprusside (SNP), a combination of 100 μM each of salicylic acid and methyl jasmonate. A mock treatment with water served as control. The treatments were carried out at different time intervals up to 24 h and incubated in a growth room at 27 ± 1 °C under 14/10 h photoperiod provided by light intensity of 30 μmol·m⁻²·s⁻¹. Samples were collected at regular intervals, quickly frozen in liquid nitrogen, and stored at −80 °C until use.

**Expression analysis of AdZADH2 using qRT-PCR**

Total RNA, isolated from control- and hormone-treated peanut samples was treated with RNase free DNase1 (Sigma-Aldrich, Carlsbad, CA, USA) to eliminate any DNA contamination and was reverse transcribed with oligo-dT primer using SMART™ MMLV Reverse Transcriptase (Clontech, Becton Dickinson, Palo Alto, CA, USA). cDNA samples were diluted fivefold and 0.5 μL of the diluted reaction mixture was taken as qRT-PCR template in a 20 μL total reaction volume containing 0.4 μM gene specific primers with10 μL SYBR Premix Ex Taq with ROX (Takara Bio Inc., Kusatsu, Shiga, Japan) and the samples were appraised in three technical replicates. PCR analysis was carried out in Realplex amplifier (Eppendorf, Hamburg, Germany) with the following cycle parameters: 95 °C for 5 min; 40 cycle of 95 °C for 20 s, 58 °C for 20 s, 72 °C for 20 s followed by melting curve. Gene specific primers were designed from 3’ UTR of *AdZADH2* (Table S1). Polyubiquitin (UBI1) and alcohol dehydrogenase III (*ADH3*) were used as internal controls for calculating relative quantification of gene expression [25,26]. Relative fold change in RNA expression was estimated using ΔΔCT method [24].

**Results and discussion**

**AdZADH2 sequence analysis**

A partial fragment of a zinc-binding alcohol dehydrogenase 2 was differentially upregulated in a cDNA-AFLP analysis of an interaction between *Arachis diogoi* and the late leaf spot pathogen *P. personata* [17]. It was made full length using 3'/5' RACE. The cDNA of *AdZADH2* comprised an ORF of 1902 bp (Fig. S1) and codes for a polypeptide of 633 amino acids (GenBank Accession number KT321126). Its upregulation under fungal treatment has been revalidated in a comparative real time PCR analysis of the resistant wild peanut and its susceptible cultivated peanut counterpart (Fig. 1C); its upregulation under pathogen challenge was observed only in the wild peanut.

Multiple sequence alignment and phylogenetic analyses displayed its close relation in plants to soybean predicted zinc-binding ADH2-like protein (different from ADH2 studied [27–29]) with 85% sequence similarity (Fig. S2a). *AdZADH2* shows BLASTP specific hit of Mgc45594_like in MDR domain region (Table S3) with an undetermined function. Psort ([http://psort.hgc.jp/form.html](http://psort.hgc.jp/form.html)) identifies *AdZADH2* as a resident of peroxisomes (probability of 85.2%); moreover, PredPlantPTS1 ([http://ppp.gobics.de](http://ppp.gobics.de)) predicted the presence of a C-terminal PTS1 domain (tripeptide AKL) for peroxisome targeting (probability 100.0%). This C-terminal signal for peroxisome translocation is absent in previously studied ADH proteins. Moreover, we made multiple sequence alignment between *AdZADH2* with some previously studied ADH proteins using network protein sequence analysis (NPS, [https://npsa-prabi.ibcp.fr/cgi-bin/align_multalin.pl](https://npsa-prabi.ibcp.fr/cgi-bin/align_multalin.pl), [30]. Interestingly, the NADB Rossmann domain was absent in other plant ADH proteins studied so far (Fig. S4) showing that it is a novel ADH2. The predicted molecular weight and pI of *AdZADH2* protein were found to be totally different from ADH proteins studied earlier (Table S3).

A protein BLAST analysis of *AdZADH2* revealed two conserved domains, a NADB Rossmann domain and MDR domain (Fig. S2A,B). These domains are reportedly involved in various activities such as oxidoreductases, catalysis, and enzymatic reactions providing catalytic and structural stability to the protein [3,27]. Rossmann fold in NAD-dependent dehydrogenases found in many protein superfamilies (mostly oxidoreductases). It decides the specificity of hydride transfer thereby providing NAD or NADP as a cofactor to MDR domain for interconversion of ethanol and acetaldehyde. Interestingly NADB Rossmann domain of *AdZADH2* was not much conserved as it showed just 83%, 72%, and 64% homology with NADB domains soybean (*Glycine max*; XP_003518845.1), tobacco (*Nicotiana tomentosiformis*; XP_009612756.1) and *Arabidopsis* (*Arabidopsis thaliana*; NP_175390.2), respectively; however, MDR domain was found to be highly conserved (Fig. S2).

Plant alcohol dehydrogenases (ADH) have been well characterized for their role in cell survival under hypoxic and anaerobic conditions. During anaerobic glycolysis i.e., fermentation, ADH diverts the formation of lactic acid from pyruvate to less toxic...
and more diffusible ethanol, thereby helping cells to survive in the absence of oxygen required for normal respiration. Available evidences suggest that plant ADH gene family comprises survivors of multiple rounds of gene expansion and contraction resulting in gains in roles like production of characteristic scents that act to attract animals, pollinators, or agents of seed dispersal and to protect plants against herbivores besides anaerobic fermentation [1,2].

**Conditional expression of AdZADH2 in tobacco results in HR-like cell death**

Transient expression of AdZADH2 in detached leaves of tobacco under an estradiol inducible promoter (XVE) [31,32] resulted in hypersensitive response (HR) like cell death in the infiltrated area 48–72 h postestradiol treatment (Fig. 2A,B). Induced expression of AdZADH2 was observed to be strong at 24 and 48 h postinfection (hpi). This strong expression of AdZADH2 was found to be associated with enhanced expression of defense-related genes (NtPR1a, NtPR1b, and NtPR5-TLP). Along with the expression of genes for pathogenesis-related proteins, marker genes for cell death such as NtPAT3, HMG-CoA R, HSR203J, and HIN1 and antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (Mn-SOD) also got upregulated in the infiltrated regions analyzed by quantitative RT-PCR up to 48 h postestradiol application (Fig. 3). HMG-CoA R, HSR203J, and HIN1 were found to be co-upregulated at 24 and 48 h of treatment. HIN1 and HSR203J have been shown to be closely associated with cell death in incompatible interactions in tobacco and were used as markers for HR [32–34]. Along with HR marker genes, we have
also observed the upregulation of \textit{PAT3} gene; transient expression of a patatin like phospholipase in tobacco resulted in the accumulation of fluorescent phenolics, pathogenesis-related proteins CaPR1, CaSAR82A, and ROS leading to cell death in pepper leaves [35]. Expression profiling postfungal treatment in \textit{Populus trichocarpa} showed possible involvement of \textit{CAD}/CAD-like genes in plant development and defense against various pests and pathogens [36]. The accelerated cell death gene, \textit{acd11} of \textit{Arabidopsis} constitutively expresses the defense-related genes that are associated with hypersensitive response normally triggered by avirulent pathogens [37].

There is evidence of the involvement of ADH in plant fungal interactions through the activation of fermentative pathway. Pathogen infection, particularly biotrophic pathogens, leads to a condition of reduced oxygen pressure that leads to a fermentation pathway and the development of reactive oxygen species. Under such conditions, upregulation of ADH has been reported in biotrophic pathogen infections. Proels et al., [38] have observed that the barley seedlings
Fig. 3. Relative expression of defense-associated genes and cell death related genes in leaves transiently expressing pER8:AdZADH2, pER8:AdZADH2-D1 and pER8:AdZADH2-D2 in relation to control (pER8) after estradiol application at 0, 24, and 48 h. Quantitative RT-PCR analysis was performed using total RNA from samples collected at various time points. Relative expression was calculated by comparative ΔΔCt method. Statistical analysis was performed with student’s t-test, asterisks indicate upregulated expression relative to control samples (*P < 0.05, **P < 0.001, ***P < 0.0001). Gene specific primers were used for amplification of different genes with Actin serving as internal reference gene. The primers sequence used in the study were given in Table S2.
Novel AdZADH2 of Arachis diogoi induces cell death

D. Kumar et al.

- treated with the biotrophic pathogen, *Blume-ria graminis* f.sp hordei upregulated ADH1 and ADH2, but not ADH3. It has been surmised that the pathogen infection might reduce photosynthetic activity in the infected tissues leading to reduced oxygen pressure resulting in a condition similar to anoxia and inducing the fermentation pathway to compensate the reduced energy levels. Moreover, ethanolic fermentation (a major means of ATP production under anaerobic condition in plants) leads to the accumulation of high levels of acetaldehyde, which is a strong cell toxin. It binds to nucleic acids and proteins forming stable acetaldehyde-protein adducts, eventually leading the cell toward PCD [39,40]. Uehara et al., [41] analyzed the differentially upregulated *ADH* gene in resistant and susceptible cultivars of tomato in response to the challenge from the cyst nematode and observed that there was differential upregulation of the *ADH* transcripts; incompatible interaction showed upregulation and compatible interaction evidencing downregulation of *ADH*. Hren et al., [42] observed the induced expression of sucrose synthase and alcohol dehydrogenase in resistant varieties of grapevine infected by phytoplasm with the conversion of sucrose to alcohol through alcohol fermentation.

Cell death phenomenon was associated with the enhanced expression of defense-related genes, cell death marker genes and antioxidant enzymes (Fig. 3). Cell death was quantified by using Evans Blue and an ion leakage experiment [43]. Cell death with enhanced electrolyte leakage was significantly high in pER8: AdZADH2 regions in comparison to the empty vector infiltrated areas (Fig. 2D,E). The AdZADH2 protein carries two domains, a NADB Rossmann superfamily domain and a MDR Superfamily domain. Furthermore, to see if the cell death could be specifically associated with one of the two domains, we have cloned these domains [as both of them carry separate start (Met) codons] separately under the estradiol inducible promoter in pER8 as NADB Rossmann domain D1 (pER8:AdZADH2-D1) and MDR domain D2 (pER8: AdZADH2-D2). We found that after chemical induction, infiltrated area expressing MDR domain (pER8: AdZADH2-D2) showed significantly enhanced cell death in comparison to NADB Rossmann domain (pER8:AdZADH2-D1) (Fig. 2C). In addition, elec-

![Fig. 4. RNA expression of zinc-binding alcohol dehydrogenase 2 in response to stress hormones. A quantitative RT-PCR was performed using total RNA isolated from samples collected at different time points (in hours) involving treatments with Salicylic acid (SA), Methyl jasmonate (MeJA), Salicylic acid and Methyl jasmonate together, Abscisic acid (ABA), Ethephon and Sodium nitroprusside (SNP). Relative transcript expression was calculated by comparative ΔΔCt method. For qPCR analysis primers were designed from 3’ UTR of *AdZADH2*. Data were normalized to the Polyubiquitin (UBI1) and Alcohol dehydrogenase-3 (*ADH-3*) level as internal reference genes and plotted from three independent experiments. Statistical analysis was performed with student’s t-test, asterisks indicates significant up-accumulation in relative to mock sample (*P < 0.05, **P < 0.001).](image-url)
trollyte leakage analysis and cell death quantified by Evans Blue also supported this observation, where enhanced ion leakage and cell death have been observed with MDR domain in comparison to NADB Rossmann domain (Fig. 2D,E). However, the level of cell death induced by individual domains was less when compared with the complete protein, AdZADH2. Taken together, these results imply that transient expression of AdZADH2 induces cell death and MDR domain played a major role in this phenomenon probably in an interaction with the NADB domain (the expression of which also induced cell death, though not that extent as the MDR domain). Rossmann fold being a NAD-dependent dehydrogenase dictates the specificity of hydride transfer thereby providing NAD or NADP as a cofactor to MDR domain for interconversion of ethanol and acetaldehyde. Therefore, in the absence of Rossmann fold, MDR domain is probably not able to produce the same level of HR-like cell death as produced when both domains are present as in AdZADH2 owing to the reduced availability of NAD or NADP as a cofactor. Quantitative expression analysis shows overexpression of defense related genes, HR marker genes and ROS generated enzymes upon transient overexpression of pER8:AdZADH2 (Fig. 3); however, the relative fold changes were significantly less when these two domains were overexpressed individually. Moreover, changes in the expression level of HR marker genes viz. HIN1, HSR203J and HMG-CoA R were not significant when Rossmann fold was overexpressed alone.

Expression analysis of AdZADH2 in response to various signaling molecules
Salicylic acid and methyl jasmonate are the important signaling molecules in systemic acquired resistance (SAR) and wound signaling, respectively, and the expression of AdZADH2 showed strong accumulation in transcripts in salicylic acid and methyl jasmonate-treated samples at early time points. AdZADH2 appeared to be regulated primarily by salicylic acid as the induced expression at 3 h stage in the combination treatment of SA and MeJA exhibited down regulation of the transcripts in comparison to 3 h MeJA treatment. Nitric oxide (NO) is an emerging essential component of plant defense signaling and SNP treatment. An interaction between hydrogen peroxide and nitric oxide is shown to be associated with several forms of cell death [52,53]. AdZADH2 appears to support nitric oxide (NO) mediated HR cell death; NO production leads to the production of H$_2$O$_2$ (instead of ROS), which in turn results in HR-mediated PCD [54]. Several lines of evidence have shown that the early nitric oxide (NO) burst in host plant cells after pathogen attack functions as a major defense response associated with resistance in plant pathogen interaction [55]. NO is an interesting molecule that is used by microorganisms in pathogenesis and host cells to activate the immune system to counter the pathogen challenge [56,57]. Thus, the significant upregulation of AdZADH2 by the resistant host against the LLS pathogen attack under SNP treatment could be justified as a resistance response.

Conclusion
 Peroxisomal resident novel AdZADH2 protein was upregulated in A. diogoi in treatment with P. personata. Conditional overexpression of AdZADH2 in tobacco leaf under an estradiol inducible promoter (XVE) resulted in HR-like cell death associated with an enhanced electrolyte leakage and enhanced expression of defense-related genes (NtPAT3, NtPR1a, NtPR1b, and NtPR5), marker genes for cell death such as HMG R, HSR203J, and HIN1 and antioxidant enzymes such as CAT, APX, and SOD. The expression of AdZADH2 was upregulated post-SA, JA, and
SNP treatments in *A. diogoi*. This report shows involvement of Zn containing ADH protein having NADB Rossmann fold and MDR domain in HR-like cell death in plants. Cell death activities are not domain additive, but are probably a result of interaction between these two domains; cell death was significantly decreased when the two domains are individually overexpressed.

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**Author contributions**

PBK and DK conceived and designed the project, SR, DK, and NKS acquired the data, where NKS identified full gene sequence of AdZADH2 by RACE PCR. SR and DK performed experiments related to HR and real-time expression studies. PBK, SR and DK analyzed, interpreted the data, and wrote the paper.

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Novel AdZADH2 of *Arachis diogoi* induces cell death

D. Kumar et al.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Table S1.** Oligonucleotides sequence used in the study.

**Table S2.** Primers and their sequences used in study for semi-quantitative and quantitative PCR Analysis.

**Table S3.** A list of previously studied ADH genes.

**Figure S1.** Cloning of AdZADH2 from *Arachis diogoi*.

**Figure S2.** Multiple sequence alignment of the deduced amino acid sequence of AdZADH2 with closely related uncharacterized Zinc-binding alcohol dehydrogenase sequences.

**Figure S3.** Phylogenetic relationship of AdZADH2 with other zinc-binding alcohol dehydrogenase family members.

**Figure S4.** Multiple sequence alignment of the deduced amino acid sequence of AdZADH2 with some previously studied zinc-binding alcohol dehydrogenase sequences from other organisms was done using default parameters of network protein sequence analysis (NPS). NADB Rossmann fold is absent in previously studied ADH proteins; moreover MDR domain of AdZADH2 shows no significant homology with other ADH proteins in the genes used in the alignment were from *Zea mays* (ZmADH1, ZmADH2), *Oryza sativa* (OsADH1), *Solanum lycopersicum* (SlADH1), *Arabidopsis thaliana* (AtADH1, AtADH2), *Glycine max* (GmADH1, GmADH2), and *Lotus japonicus* (LjADH1), respectively. Accession numbers are given in Table S3.

**Figure S5.** Cloning of NADB Rossmann domain and MDR superfamily domain.

**Figure S6.** Transient constitutive expression of AdZADH2 induced cell death in tobacco leaf upon constitutive expression under 35S promoter.