Histone deacetylase HD2 interacts with ERF1 and is involved in longan fruit senescence

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Abstract

Histone deacetylation plays an important role in epigenetic control of gene expression. HD2 is a plant-specific histone deacetylase that is able to mediate transcriptional repression in many biological processes. To investigate the epigenetic and transcriptional mechanisms of longan fruit senescence, one histone deacetylase 2-like gene, DlHD2, and two ethylene-responsive factor-like genes, DlERF1 and DlERF2, were cloned and characterized from longan fruit. Expression of these genes was examined during fruit senescence under different storage conditions. The accumulation of DlHD2 reached a peak at 2 d and 30 d in the fruit stored at 25 °C (room temperature) and 4 °C (low temperature), respectively, or 6 h after the fruit was transferred from 4 °C to 25 °C, when fruit senescence was initiated. However, the DlERF1 transcript accumulated mostly at the later stage of fruit senescence, reaching a peak at 5 d and 35 d in the fruit stored at 25 °C and 4 °C, respectively, or 36 h after the fruit was transferred from low temperature to room temperature. Moreover, application of nitric oxide (NO) delayed fruit senescence, enhanced the expression of DlHD2, but suppressed the expression of DlERF1 and DlERF2. These results indicated a possible interaction between DlHD2 and DlERFs in regulating longan fruit senescence, and the direct interaction between DlHD2 and DlERF1 was confirmed by yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. Taken together, the results suggested that DlHD2 may act with DlERF1 to regulate gene expression involved in longan fruit senescence.

Key words: ERFs, fruit senescence, HD2, histone deacetylase, longan.

Introduction

The regulation of gene expression at the transcription level underlies many biological aspects including growth and development, metabolic and physiological balances, and responses to the environmental stimuli (Vlachonasios et al., 2003). Gene expression depends on not only DNA sequences such as promoters and cis-acting regulatory elements, but also epigenetic factors such as histone modifications and chromatin remodelling. In eukaryotes, post-translational modification of histone plays an important role in gene regulation (Turner, 2000; Lagaçé et al., 2003). A number of post-translational modifications of histone have been observed, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation (Strahl and Allis, 2000). All histone modifications are removable, which may therefore provide a versatile way for regulating gene expression during plant development and response to environmental stimuli. Acetylation of the histones catalysed by histone acetyltransferases (HATs) is generally associated with increased...
transcriptional activation, whereas deacetylation of histones by histone deacetylases (HDACs) is correlated with transcriptional repression (Berger, 2002; Sridha and Wu, 2006).

Three types of HDACs, namely reduced potassium dependency 3 (RPD3), histone deacetylase 1 (HD1), and silent information regulation 2 or sirtuin 2 (Sir2), have been found in plants, yeast, and animals (Pandey et al., 2002; Yang and Seto, 2003). In addition, plants contain a unique type of HDACs called histone deacetylase 2 (HD2), which is unrelated to the RPD3, HD1, and Sir2 HDAC types (Sridha and Wu, 2006). The first HD2 was isolated as an acidic nucleolar phosphoprotein from maize embryo (Lusser et al., 1997), suggesting a possible role for HD2 in the expression of rRNAs. Later, four HD2 proteins, AtHD2A, AtHD2B, AtHD2C, and AtHD2D, were identified in Arabidopsis (Wu et al., 2006). AtHD2A and AtHD2B were shown to be involved in establishing leaf polarity in Arabidopsis (Ueno et al., 1997) while the Arabidopsis AtHD2C gene played a role in abscisic acid (ABA) response and abiotic stress (Sridha and Wu, 2006). ScHD2a, an orthologue of AtHD2A in Solanum lycopersicum (a wild species related to potato), was strongly induced in ovules after fertilization (Lagaçe et al., 2003). In addition, HvHDAC2-1 and HvHDAC2-2 from barley were found to respond to plant stress-related hormones, such as jasmonic acid (JA), ABA, and salicylic acid (SA) (Demetriou et al., 2009). Collectively, these findings suggest that HD2-type HDACs play an important role in plant development and stress response. Furthermore, HD2 may modulate gene expression in a complex containing RPD3-like HDACs such as AtHD6A and AtHD1, although it is yet to be determined whether any HD2 members interact with RPD3-like proteins (Chen and Tian, 2007). Recently, DNA methyltransferase 2 (AtDNMT2) interacting with AtHD2s has been identified in Arabidopsis (Song et al., 2010). It has been demonstrated that chromatin structure including the structure imposed by the nucleosome implies that transcription factors (TFs) work in a complex with RPD3-like HDACs such as AtHD2. It has been shown that expansin (EXP), xyloglucan endotransglucosylase (XET), and endo-β-1,4-glucanase (EGase) related to cellular wall metabolism are involved in longan fruit senescence (Zhong et al., 2008; Xiao et al., 2009). However, little attention has been paid to the epigenetic and transcriptional mechanisms of longan fruit senescence. In this study, one HD2 gene and two ERF genes were isolated from longan fruit. The expression patterns of these genes in relation to senescence of longan fruit stored under various conditions, and their responses to NO treatment, were investigated. Moreover, the direct interaction between HD2 and ERF1 was detected by yeast two-hybrid assay and the bimolecular fluorescence complementation (BiFC) assay, suggesting that DIHD2 may mediate longan fruit senescence by interacting with DIERF1.

Materials and methods

Plant materials

Longan (N. chinensis) fruit at physiological maturity (~90 d after anthesis), exhibiting yellow-brown colour in peel and optimal eating quality of the aril (Jiang et al., 2002), were...
harvested, then transported to the laboratory within 2 h, and finally selected for freedom from visual defects and for uniformity of weight, shape, and maturity.

Treatments

The selected longan fruit were divided randomly into four groups and then placed into unsealed plastic bags (0.04 mm in thickness). Each bag contained 50 individual fruit and 14 bags were used as one group. For NO treatment, the fruit from Groups 1 and 2 were exposed to air (control) or 40 µl l⁻¹ NO for 4 h in six closed chambers (5.0 l) at 25°C. After these treatments, the fruit were placed into unsealed plastic bags and then stored at 25°C (room temperature) for 5 d until the control fruit were completely senescent. Fruit were sampled daily. The fruit from Group 3 were stored at 4°C (low temperature) for 45 d and then sampled at 5 d intervals, while the fruit from Group 4 were stored at 4°C for 40 d and then transferred to 25°C for further storage, and the fruit were sampled at 0, 6, 12, 24, 36, and 48 h, respectively. Each sampling contained 60 individual fruit withdrawn randomly from six bags. The whole aril of 20 individual fruit were mixed thoroughly as one replicate, frozen in liquid nitrogen, and stored at −80°C until use. All assessments were conducted in three biological replicates.

Evaluation of fruit senescence

The aril breakdown index was used to indicate the longan fruit senescence. The index was measured by determining the area ratio of the aril which had broken down to the whole aril using 30 individual fruit per sample, according to the methods of Zhong et al. (2008) and Xiao et al. (2009).

RNA extraction and isolation of longan DIHD2, DIERF1, and DIERF2 full-length cDNAs

Total RNA from longan fruit was extracted using the hot borate method of Wan and Wilkins (1994). Frozen aril tissues (10 g) were ground to a fine powder in a mortar using a pestle in the presence of liquid nitrogen. Following the RNA extraction, potentially contaminated RNA was eliminated by the treatment with DNase I digestion using the RNase-free kit (Promega, Madison, WI, USA). The DNA-free total RNA was then used as template for reverse transcription-PCR (RT-PCR). The first-strand cDNA of the product was subjected to PCR amplification. To isolate HD2 cDNA from longan fruit, two synthetic degenerate oligonucleotide primers were designed with regard to the N-terminal (METFWGVE) and the internal peptide sequences (HVATPHP) of the HD2 protein (Aravind and Koonin, 1998). Degenerate primers for the ERF were designed based on the report of Tournier et al. (2003). The isolated fragments were cloned into the pMD20-T vector (TaKaRa, Dalian Division), sequenced, and finally compared with the database sequence using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Next, 3′- or 5′-rapid amplification of cDNA ends (RACE)-PCR was performed using cDNA end amplification kits (TaKaRa, Dalian Division) according to the manufacturer’s protocol. In order to amplify 3′ and 5′ end fragments, the specific primers were designed based on the nucleotide sequences of the cDNA fragments already cloned by RT-PCR. The 3′- and 5′-RACE-PCR products were cloned and sequenced as described above. The primer sequences are visualized by TreeView software. The theoretical isoelectric points (pIs) and mass values for mature peptides were calculated using the PeptideMass program (http://us.expasy.org/tools/peptide-mass.html).

Transcriptional activation analysis in yeast cells

The open reading frames (ORFs) of DIHD2, DIERF1, and DIERF2 were amplified by PCR with gene-specific primers (listed in Supplementary Table S2 at JXB online) and subcloned into pGBK7T (Clontech, USA) (Supplementary Fig. S1). According to the protocol of the manufacturer, pGBK7-T-DIERF1, pGBK7-T-DIERF2, pGBK7-T-DIHD2, the positive control pGBK7-T53+pGADT7-T, and the negative control pGBK7T plasmids were each used to transform the AH109 yeast strain. The transformed strains were streaked onto SD/-Trp or SD/-Trp–His–Ade plates. The transactivation activity of each protein was evaluated according to their growth status and the β-galactosidase activity.

Subcellular localization analysis

The coding region sequences of DIERF1, DIERF2, and DIHD2 without the stop codon were amplified by PCR (the primers are listed in Supplementary Table S3 at JXB online) from the full-length clone of pMD-DIERF1, pMD-DIERF2, and pMD-DIHD2, and subcloned into the pUC-GFP vector, in-frame with the green fluorescent protein (GFP) sequence, resulting in the 35S::gene-GFP vectors under the control of the Cauliflower mosaic virus (CaMV) 35S promoter (Supplementary Fig. S2). The fusion constructs and the control GFP vector were introduced into onion epidermal cells by particle bombardment using a Bio-Rad (Hercules, CA, USA) Biologic Particle Delivery System PDS-1000. GFP fluorescence was observed with a laser scan confocal microscope. All transient expression assays were repeated at least three times.

Northern blot analysis

Total RNA (10 µg) was separated on a 1.2% agarose-formamide gel and capillary blotted onto a positively charged nylon membrane (Biodyne B, 0.45 µm, PALL Co., Sarasota, FL, USA). The RNA was fixed to the membrane by baking for 2 h at 80°C and then cross-linked to the membranes using an ultraviolet cross-linker (Armelham Biosciences, Piscataway, NJ, USA). The membranes were pre-hybridized for >3 h in SDS buffer solution containing 50% (v/v) deionized formamide, 5× SSC, 7% SDS, 2% blocking reagent (Roche Diagnostics, Mannheim, Germany), 50 mM sodium phosphate (pH 7.0), and 0.1% N-lauroylsarcosine (w/v). Hybridization was performed overnight in the same buffer solution containing the gene-specific digoxigenin (DIG)-labelled probe at 45°C. The probe was prepared with a DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instruction. The probes were synthesized from the 3′-untranslated regions of the genes and each membrane was only hybridized once with each probe to ensure that the hybridization was specific and reliable. Following hybridization, membranes were washed twice for 10 min with 2× SSC containing 0.1% SDS at 25°C, followed by washing twice for 30 min in 0.1× SSC containing 0.1% SDS at 62°C. The signals were detected with chemiluminescence using CDP-Star™ (Roche Diagnostics) as described by the manufacturer. The most stable reference gene, iron superoxide dismutase (FeSOD) (Lin and Lai, 2010), was used as an internal control. Membranes were scanned with a densitometer (Bio-Rad Fluor-S Multimager) and then the hybridization signals were quantified by using the Bio-Rad Quantity One software. The relative expression abundance of each gene was expressed as the ratio of target gene intensity to FeSOD intensity. The specific primers used for synthesis of the DIG-labelled probes are available in Supplementary Table S4 at JXB online.
Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the Matchmaker GAL4-based two hybrid system 3 (Clontech, USA). The ORFs of DIHD2, DIERF1, and DIERF2 were amplified by PCR and subcloned into pGBK7 and pGADT7 vectors as bait and prey, respectively (Supplementary Fig. S3 at JXB online). The primers used for PCR cloning of the cDNAs are listed in Supplementary Table S5. Construct pairs pGBK7-T53+pGADT7-T (positive control), pGBK7-Lam+pGADT7-T (negative control), pGBK7-DIHD2+pGADT7-DIERF1, pGBK7-DIHD2+pGADT7-DIERF2, and pGBK7-DIHD2+pGADT7-DIHD2 were transformed into the yeast strain AH109 by the lithium acetate method. Transformed yeast cells are first grown on a minimal medium/–Leu–Tryp according to the manufacturer’s instructions (Clontech), and then plated onto a minimal medium/–Leu–Trp–His–Ade containing 5 mM 3-amino-1,2,4-triazole (3-AT) at 30 °C to test the possible interactions between DIHD2 and DIERFs.

BiFC assay in Arabidopsis mesophyll protoplasts

The vectors used in the BiFC assay (pUC-pSPYNE or pUC-pSPYCE) were obtained from the laboratory of Harter and Kudla (Walter et al., 2004). For generation of the BiFC vectors (Supplementary Fig. S4 at JXB online), the full-length coding sequence of DIERF1 was fused with the N-terminal fragment of yellow fluorescent protein (YFP) in the pUC-pSPYNE vector to form the DIERF1-pSPYNE construct, while the full-length coding sequence of DIHD2 was cloned into pUC-pSPYCE as a fusion with the C-terminal fragment of YFP to produce the DIHD2-pSPYCE construct. Both empty vectors (pUC-pSPYNE/pUC-pSPYCE) and expression of DIERF1 alone (DIERF1-pSPYNE/pUC-pSPYCE) were used as negative controls, while the bZIP63-pSPYNE and bZIP63-pSPYCE vectors were used as a positive control (Walter et al., 2004). The resulting constructs were used for transient assays by polyethylene glycol (PEG) transfection of Arabidopsis protoplasts isolated from 4-week-old wild-type (Columbia) plants according to previously reported procedures (Yoo et al., 2007). mCherry-Vir2D2Ls was included in each transfection to serve as a control for successful transfection as well as for nuclear localization (Lee et al., 2008). Transfected cells were imaged using a TCS SP5 Confocal Spectral Microscope Imaging System (Leica), with an argon blue laser at 488 nm, a beamsplitter for excitation at 500 nm, and a spectral detector set between 515 nm and 540 nm. The primers used in BiFC assay are also listed in Supplementary Table S5 at JXB online.

Results

Isolation and sequence analysis of full-length cDNA of DIHD2, DIERF1, and DIERF2

One HD2-like and two ERF-like full-length cDNAs were isolated from longan aril and designated as DIHD2, DIERF1, and DIERF2, respectively. DIHD2 contained an ORF of 931 bp. It encoded a putative protein of 306 amino acids, with a predicted mol. wt of 32.8 kDa and a calculated pl of 4.86. Significant homology was identified between DIHD2 and histone deacetylase HD2 from other organisms, based on their deduced protein sequences. The deduced DIHD2 protein contained all the structural features of the plant-specific HD2-type proteins (Aravind and Koonin, 1998), such as an N-terminal domain with the invariable pentapeptide (MEFWG), a central region containing an extended acidic domain, required for repression, followed by a central acidic region rich in glutamatic and/or aspartic acid, and a single C2H2-type zinc finger domain in the C-terminus, which may enable high affinity DNA binding or mediate protein–protein interactions (Dangl et al., 2001; Zhou et al., 2004) (Supplementary Fig. S5 at JXB online). A BLAST search of GenBank revealed that DIHD2 shared 55% identity with RchHD2a (XP_002527449) from bean and 53% identity with NtHD2a (ACZ54945) from tobacco at the protein level. Phylogenetic analysis further showed that DIHD2 was closely related to HD2a from Ricinus communis (Supplementary Fig. S6).

DIERF1 had an ORF of 813 bp, encoding a predicted polypeptide of 270 amino acids, with a predicted mol. wt of 29.8 kDa and a calculated pl of 5.62, while DIERF2 exhibited an ORF of 966 bp, encoding 321 amino acids with a predicted mol. wt of 35.3 kDa and a calculated pl of 4.96. The deduced amino acid sequences of the two DIERFs comprised a conserved DNA-binding ERF/AP2 domain, which is a typical characteristic of the plant ERF gene families (Supplementary Fig. S7 at JXB online). In addition, both DIERF1 and DIERF2 contained two key amino acid residues, the 14th alanine (A14) and the 19th aspartate (D19), which were reported to contribute to a functional GCC-box binding activity in many ERFs (Sakuma et al., 2002). Phylogenetic analysis also indicated that the ERF family proteins were classified into four groups, Class I, II, III, or IV (Supplementary Fig. S8). The first three groups were previously identified according to their functions and structures (Fujimoto et al., 2000). Class IV, a new member of the ERF subfamily containing a conserved MCGGAII signature sequence in the N-terminus, was later added to the classification (Tournier et al., 2003). As shown in Supplementary Fig. S8, DIERF1 belonged to the Class III ERFs (Fujimoto et al., 2000; Nakano et al., 2006). The ERF genes in this group had a CMIX-2 motif and, thus, were subclassified into three types based on the different constituents of their putative mitogen-activated protein (MAP) kinase phosphorylation sites, the CMIX-5 motif, and the CMIX-6 motif (Nakano et al., 2006). DIERF1 had a higher similarity to NtERF4 and SIERF4 than to other types of ERFs, in which both the CMIX-5 motif and the CMIX-6 motif were found at the C-terminus (Supplementary Fig. S9). However, DIERF2 belonged to Class IV ERFs (Tournier et al., 2003; Nakano et al., 2006), characterized by a conserved N-terminal signature and the MCGGAII/L motif (CMVII-1 motif) (Supplementary Fig. S10). Overall, these results suggested that DIERF1 and DIERF2 might exhibit diverse functions.

DIERF1 showed transcriptional activation activity in yeast

To investigate the transcriptional activities of DIHD2, DIERF1, and DIERF2, a transient expression assay using a GAL4-responsive reporter system was performed. For the assay of transcriptional activity, the entire ORF of DIHD2, DIERF1, or DIERF2 was cloned into the same pGBK7 vector (Supplementary Fig. S1 at JXB online). The yeast strain AH109 harbouring two reporter genes, lacZ and His3, was transformed with the fusion plasmids pGBK7-DIHD2,
pGBK7-DlERF1, and pGBK7-DlERF2, the positive control pGBK7-53+pGADT7-T, and the negative control pGBK7, respectively. As shown in Fig. 1, the transformed yeast cells harbouring pGBK7-DlERF1 and pGBK7-53+pGADT7-T (the positive control) grew well in the SD medium lacking tryptophan, histidine and adenine, and showed β-galactosidase activity, whereas the cells containing pGBK7-DlHD2, pGBK7-DlERF2, or pGBK7 (the negative control) did not grow or showed no β-galactosidase activity. These data suggested that DlERF1 functioned as a transcriptional activator in yeast.

Subcellular localization of DlHD2 and DlERFs

To validate the subcellular localization of DlHD2 and DlERF proteins, the coding regions of the DlHD2, DlERF1, and DlERF2 were fused in-frame with the GFP gene (Supplementary Fig. S2 at JXB online), and then the resulting constructs were bombarded into onion epidermal cells. As shown in Fig. 2, the fluorescence of DlERF1–GFP and DlERF2–GFP was localized exclusively to the nucleus, while the fluorescence of GFP alone was observed in the entire cells. Interestingly, the fluorescence of DlHD2–GFP was probably localized exclusively to the three nucleoli in the nucleus. In addition, a similar subcellular localization of DlHD2 was also observed in Arabidopsis mesophyll protoplasts (Supplementary Fig. S11), indicating that DlHD2 may also act directly on rRNA gene chromatin (Lusser et al., 1997).

Expression patterns of DlHD2, DlERF, and DlERF2 during senescence of longan fruit stored under different conditions

To understand the possible role of the DlHD2, DlERF1, and DlERF2 during longan fruit senescence, the expression patterns of DlHD2, DlERF1, and DlERF2 in fruit stored under different conditions, namely room temperature (25 °C), low temperature (4 °C), the transfer from low temperature to room temperature, and application of NO, were analysed. Longan fruit began to show senescence symptoms after 2 d of storage at 25 °C, as reflected by the aril breakdown index, which increased rapidly during storage, particularly after 3 d of storage (Fig. 3A). Accumulation of DlHD2 mRNA increased in the first 2 d, but decreased afterwards as the aril broke down. The mRNAs of both DlERF1 and DlERF2 showed a gradual increase accompanying the aril breakdown, especially at days 4 and 5 (Fig. 3B; Supplementary Fig. S12). As expected, NO pretreatment delayed longan fruit senescence, as the aril breakdown index of the NO-treated fruit was significantly delayed.

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Fig. 1. Transcriptional activation analysis of DlERFs and DlHD2 in yeast. DlERF1, DlERF2, and DlHD2 were each fused with the GAL4 DNA-binding domain and expressed in yeast strain AH109. The vectors pGBK7 or pGBK7-53+pGADT7-T were expressed in yeast as a negative or a positive control, respectively. Yeast clones transformed with the different vectors were grown on SD plates without tryptophan or tryptophan, histidine and adenine for 3 d at 30 °C. Transcription activation was monitored by the detection of yeast growth and β-galactosidase assay.
lower than that of the control fruit (Fig. 3A). Correspondingly, application of NO obviously enhanced the accumulation of \textit{DlHD2} mRNA but suppressed that of \textit{DlERF1} and \textit{DlERF2} mRNAs during fruit senescence (Fig. 3B; Supplementary Fig. S12 at \textit{JXB} online).

Storage at low temperature obviously delayed the appearance of fruit senescence symptoms. Aril breakdown symptoms were observed only after 25 d of storage at 4 °C and became significant after 30 d of storage (Fig. 4A). The \textit{DIHD2} transcript continuously increased within the first
30 d, with higher accumulations at days 25 and 30, then decreased gradually afterwards (days 35–45) (Fig. 4B; Supplementary Fig. S13A at JXB online). With a rapid increase in aril breakdown index, the DIERF1 transcript was first increased, especially at days 35–42 of storage, then decreased at day 45 (Fig. 4B; Supplementary Fig. S13B). However, the DIERF2 transcript decreased during the first 2–15 d, then increased and finally reached a plateau during 20–45 d of storage (Fig. 4B; Supplementary Fig. S13C).

The aril breakdown index increased markedly when fruit were removed from 4 °C to 25 °C. The index increased progressively, and then reached a maximum level at 48 h (Fig. 5A). DlHD2 transcripts decreased progressively during the whole 48 h of storage, while DIERF1 and DIERF2 transcripts increased, reached their peaks at 36 h and 24 h, respectively, and finally decreased (Fig. 5B; Supplementary Fig. S14).

Interaction between DlHD2 and DIERF1

Collectively, based on the expression characteristics of DlHD2, DIERF1, and DIERF2 in which the DlHD2 transcript obviously decreased during fruit senescence (aril breakdown) while the DIERF1 and DIERF2 transcripts increased, it is interesting to analyse the possible interaction between DlHD2 and DIERFs. DlHD2, DIERF1, and DIERF2 coding sequences were subcloned into pGADT7 and pGBK7 vectors for yeast two-hybrid assay (Supplementary Fig. S3 at JXB online). Since DIERF1 had a trans-activation activity in yeast when fused with the BD domain (Fig. 1), DIERF1 was only fused with the AD domain in the yeast two-hybrid analysis. Similar to pGBK7-T53+pGADT7-T (positive control), yeast cells co-transformed with pGBK7-DlHD2+pGADT7-DIERF1 could grow on selective medium lacking Trp, Leu, His, and Ade in the presence of 5 mM 3-AT (Fig. 6A). In contrast, yeast cells harbouring pGBK7-T7-Lam+pGADT7-T (negative control), pGBK7-DlHD2+pGADT7-DIERF2, and pGBK7-DlERF2+pGADT7-DlHD2 could not grow on the selective medium under the same condition (Fig. 6A). These results indicated that DlHD2 may physically interact with DIERF1.

The interaction between DlHD2 and DIERF1 was further tested in plant cells using BiFC. DIERF1 tagged with pSPYNE (split YFP N-terminal fragment expression, Supplementary Fig. S4 at JXB online) and DlHD2 tagged with pSPYCE (split YFP C-terminal fragment expression, Supplementary Fig. S4) were transiently co-expressed in Arabidopsis leaf mesophyll protoplasts by PEG transfection (Walter et al., 2004; Yoo et al., 2007). As shown in Fig. 6B, cells transfected with DIERF1-pSPYNE and DlHD2-pSPYCE, and the positive control exhibited YFP fluorescence. Similar results were also observed when DlHD2-pSPYNE was co-transformed with DIERF1-pSPYCE (Supplementary Fig. S16). In contrast, the combined expression of unfused pSPYNE and pSPYCE, and the expression of DIERF1 alone did not induce any YFP signals. The BiFC results not only demonstrated the in vivo interaction between the two proteins tested but also showed the specific localization of the interacting proteins in the nucleus, which is consistent with the subcellular localization of DlHD2 and DIERF1 in the nuclear compartment (Fig. 2). These results imply that the protein complex of DlHD2 and DIERF1 may function in the nucleus.

Discussion

Characterization of DlHD2 and DIERFs

Plants differ from other eukaryotes in that they possess a new HDAC family, the HD2 family (Lusser et al., 1997; Wu et al., 2003). In this study, a HD2-type HDAC gene, designated as DlHD2, was cloned and characterized from longan aril. Alignment of DlHD2 with HD2 proteins from other organisms showed that it contained all the features of HD2 proteins (Supplementary Fig. S5 at JXB online), including an invariable pentapeptide (METFAG), an acidic domain, and a single C2H2-type zinc finger domain (Lusser et al., 1997). NCBI Blast revealed that DlHD2 exhibited the highest homology with RhHD2a in castor bean, with 55% amino acid identity. Similar to ZmHD2 (Lusser et al., 1997) and AhHD2A (Earley et al., 2006), DlHD2 was also localized exclusively in the cell nucleoli (Fig. 2, Supplementary Fig. S11), indicating that DlHD2 may be involved in the regulation of rRNA genes (Lusser et al., 1997).

ERF proteins were first identified as TFs, which possess GCC-box binding activity (Ohme-Takagi and Shinozki, 1995; Böttner and Singh, 1997). Although sequence identity can be as low as 13% among the different ERFs, all ERFs exhibited a highly conserved AP2/ERF DNA-binding domain of 57–66 amino acids (Tournier et al., 2003; Cao et al., 2006). Based on the sequence features of the AP2/ERF domain, the ERF family has been categorized into four classes (Fujimoto et al., 2000; Tournier et al., 2003). In this study, two DIERF genes from longan fruit were isolated and characterized. These DIERF genes were found to fall into two different classes of the previously characterized ERF proteins (Fujimoto et al., 2000; Tournier et al., 2003). DIERF1 contains a putative MAP kinase phosphorylation site in the C-terminal region (Supplementary Fig. S9 at JXB online) and therefore belongs to Class III ERFs, which include AtERF5 and AtERF6 from Arabidopsis, and SIERF4 from tomato. AtERF5, AtERF6, and SIERF4 have been shown to function as transcription activators (Fujimoto et al., 2000; Tournier et al., 2003). Similarly, transcriptional activation and subcellular localization analysis also indicated that DIERF1 could function as a transcriptional activator (Figs 1, 2). DIERF2 was categorized as a ‘Class IV’ ERF based on the location of the putative nuclear localization signal (NLS) site and the presence of the conserved N-terminal CMVII-1 motif (MCGGAIIL) (Supplementary Fig. S10) (Tournier et al., 2003). Although the function of the motif is still unknown, it is unlikely to be required for nuclear localization or for binding to the GCC-box (Tournier et al., 2003). Further studies are needed to unravel the function of the ‘Class IV’ ERFs (El-Sharkawy et al., 2009).
Possible roles of DIHD2 and DIERFs in longan fruit senescence

Plant development is an intricate process involving a series of highly organized mechanisms, including both genetic and epigenetic regulation. There is extensive evidence to show that plant HDACs act as global transcriptional regulators to play crucial roles in a range of plant developmental processes (Hollender and Liu, 2008; Chung et al., 2009). It has been reported that the down-regulation of $AtHD1$ by antisense inhibition or T-DNA insertion in Arabidopsis can induce various developmental defects, including early senescence, serrated leaves, aerial rosettes, defects in floral organ identity, and late flowering (Wu et al., 2000a; Tian and Chen, 2001; Tian et al., 2005). In rice, overexpression of the rice HD1 homologue OsHDAC1-3 in transgenic rice resulted in an increased growth rate and altered architecture (Jang et al., 2003). However, whether epigenetic regulation is involved in fruit senescence remains unknown. In this study, the DIHD2 transcript was highly expressed before fruit senescence but decreased dramatically during senescence (Figs 3–5; Supplementary Figs S12–S14 at JXB online), indicating that DIHD2 might be involved in the regulation of the early stage of longan fruit senescence. Histone modifications including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation are important aspects of epigenetic changes, among which acetylation and deacetylation are well characterized (Xu et al., 2005; Chung et al., 2009; Yaish et al., 2011; Yu et al., 2011). In particular, the lysines residues (K) including K9, K14, K18, K23, K27, and K56 in histone H3 and K5, K8, K12, and K16 in H4, as well as other lysines in histones H2A and H2B, are specifically acetylated or deacetylated by various HATs or HDACs within a given histone (Peng et al., 2008). For example, OsHDAC1 epigenetically repressed the expression of OsNAC6 by deacetylating K9, K14, and K18 in histone H3.

Fig. 3. Changes in the aril breakdown index (A), and differential expression patterns of DIHD2, DIERF1 and DIERF2 (B) in aril tissues of control and NO-treated longan fruit stored at room temperature (25 °C) for 5 d. In A, each value represents the means of three replicates, and vertical bars indicate the SE. Different letters indicate a statistical difference at the 5% level among data groups according to the Duncan’s multiple range test. In B, total RNA (10 μg per lane) was used for RNA gel blot analysis and hybridization with DIG-labelled probes. Hybridization with DIG-labelled DfFeFOD probes and ethidium bromide-stained rRNA are shown as the internal loading controls.
and K5, K12, and K16 in histone H4 during seedling root growth in rice (Chung et al., 2009). Recently, functional interplay between Arabidopsis HDAC and demethylase through HDA6 and FLD interaction in flowering control by increased levels of histone H3 acetylation and H3K4 trimethylation in FLC, MAF4, and MAF5 has been reported (Yu et al., 2011). These studies indicate that histone acetylation regulates chromatin structure and gene expression throughout the plant life cycle. In the present work, western blot analysis of histone H3 acetylation showed that levels of histone H3 acetylation in fruit stored at 25 °C increased progressively accompanying the aril breakdown, especially at days 3–5 (Supplementary Fig. S15), which was in parallel with the patterns of change of DlERF1 and DlERF2 (Fig. 3B), whereas it was the opposite of DlHD2 expression (Fig. 3B). In addition, levels of histone H3 acetylation were found to be lower in NO-treated fruit than in control fruit (Supplementary Fig. S15). These results indicate that histone H3 acetylation regulates chromatin structure and gene expression throughout the plant life cycle. In the present work, western blot analysis of histone H3 acetylation showed that levels of histone H3 acetylation in fruit stored at 25 °C increased progressively accompanying the aril breakdown, especially at days 3–5 (Supplementary Fig. S15), which was in parallel with the patterns of change of DlERF1 and DlERF2 (Fig. 3B), whereas it was the opposite of DlHD2 expression (Fig. 3B). In addition, levels of histone H3 acetylation were found to be lower in NO-treated fruit than in control fruit (Supplementary Fig. S15). These results indicate that histone H3 acetylation may be involved in the epigenetic regulation of longan fruit senescence by histone acetylation.

HD2 interacts with ERF1 to regulate fruit senescence

The ERF family of TFs, with a highly conserved signature element including an ERF domain responsible for the DNA binding (e.g. the GCC-box and DRE) activity, is essential for plant development and plant responses to different environmental stress factors (Okamuro et al., 1997; Sakuma et al., 2002; Cao et al., 2006; Nakano et al., 2006; Sharma et al., 2010). ERF genes associated with fruit ripening and senescence have been reported. For example, transcripts of tomato LeERF2 (Tournier et al., 2003) and apple MdERF1 (Wang et al., 2007) were accumulated during fruit ripening, and the expression of plum PsERF2a and PsERF2b (El-Sharkawy et al., 2009) was enhanced in flowers after fertilization, while kiwifruit AdERF4 and
**Fig. 6.** Physical interaction between DlHD2 and DlERF1 detected in yeast two-hybrid assays and in the BiFC system. (A) Interactions between DlHD2 and DlERF1 in the yeast two-hybrid assay. AH109 yeast strains were transformed with plasmids pGBK7-53+pGADT7-T (positive control), pGBK7-Lam+pGADT7-T (negative control), pGBK7-DlHD2+pGADT7-DlERF1, pGBK7-HD2+pGADT7-DlERF2, or pGBK7-DlERF2+pGADT7-DlHD2. The ability of yeast cells to grow on synthetic medium lacking tryptophan, leucine, histidine, and adenine, and containing 5 mM 3-amino-1,2,4-triazole (3-AT) was scored as a positive interaction. (B) BiFC visualization of the DlHD2 and DlERF1 interaction in transiently co-expressed *Arabidopsis* mesophyll protoplasts. DlHD2 protein was fused with the C-terminus of YFP and DlERF1 protein was fused with the N-terminus of YFP. The mCherry-VirD2-NLS was included in each transfection to serve as a control for successful transfection as well as for nuclear localization. Empty vectors (pUC-pSPYNE/pUC-pSPYCE) and expression of DlERF1 alone (DlERF1-pSPYNE/pUC-pSPYCE) were used as negative controls, while the *Arabidopsis* nuclear protein bZIP63 served as the positive control. The length of the bar is indicated in the photographs.
AdIERF6 were markedly stimulated at the later fruit senescence stage (Yin et al., 2010). Similarly, the DIERF1 and DIERF2 transcripts were markedly accumulated at the beginning of longan fruit senescence, and then reached higher levels at the later stage of senescence (Figs 3–5; Supplementary Figs S12–S14 at JXB online). In addition, NO is a simple diatomic free radical gas and can act as a signalling molecule (Guo and Crawford, 2005). Leshem and Pinchasov (2000) suggested that there may be an antagonistic effect of NO against ethylene during fruit maturation and senescence. As shown in Fig. 3, NO pre-treatment could effectively delay fruit senescence and suppress the expression of DIERF1 and DIERF2. These results indicated that DIERF genes were positively involved in regulating longan fruit senescence. ERFs can regulate the target gene expression in the ethylene signal transduction pathway or might relate to cellular wall metabolism by binding to the GCC-box in the promoter region. These target genes in turn may regulate the firmness, aroma, taste, colour, and shelf life of harvested fruit (Nath et al., 2006). Interestingly, AdIERF0 significantly suppressed the activity of the AdXET5 promoter, which does not have a GCC-box or DRE (Yin et al., 2010). In tomato, Pti4, an ERF-like TF, could also regulate defence-related gene expression via either GCC- or non-GCC-boxes (Chakravarthy et al., 2003). In previous studies, it was demonstrated that cellular wall-modifying genes such as DIEXP, DIXET, and DIEGase genes were associated with longan aril breakdown (Zhong et al., 2008; Xiao et al., 2009). Further research is required to determine whether DIERF genes are involved in fruit senescence via regulating DIEXP, DIXET, or DIEGase genes.

Interaction of DIHD2 with DIERF1 in longan fruit senescence

Both histone acetylation and deacetylation are promoter dependent, locus specific, and reversible, which may therefore provide a versatile way to regulate gene expression during plant development or the plant response to environmental stimuli (Tian et al., 2005; Yu et al., 2011). It is generally accepted that HATs can be recruited through transcriptional activators whereas the HDACs interact with transcriptional repressors. For instance, Arabidopsis HAT protein could interact with the transcriptional activator CBF1 to modulate cold-regulated gene expression (Stocker et al., 2001). On the other hand, the Class I RPD3 family of HDACs, HDA19, may act in a protein complex with AtERF7, a transcription repressor (Song et al., 2005), to regulate abiotic stress response genes. It was found that AtERF7 interacted with the Arabidopsis homologue of a human global co-repressor of transcription, AtSin3 (Silverstein and Ekwall, 2005), which, in turn, may interact with HDA19, indicating that AtERF7, AtSin3, and HDA19 can form a transcriptional repressor complex to regulate the ABA- and drought-related response in Arabidopsis (Song et al., 2005). HDACs seem to interact specifically with transcriptional repressors, but they also interact with certain transcriptional activators. For example, Arabidopsis HDA19 interacted with the transcriptional activators WRKY38 and WRKY62 (Kim et al., 2008). Overexpression of HDA19 specifically prevented activation of transcription by WRKY38 and WRKY62 and enhanced plant resistance to pathogens (Kim et al., 2008). Interestingly, it was also found that longan DIHD2 played a role in regulating longan fruit senescence opposite to those of DIERF1 and DIERF2 in the present study. Moreover, DIHD2 could interact with the transcriptional activator DIERF1 (Fig. 6; Supplementary Fig. S16 at JXB online), which suggested that they might act in the same protein complex to regulate gene expression involved in fruit senescence. Further experiments such as pull-down and co-immunoprecipitation assays will be needed to confirm the interaction of DIHD2 with DIERF1. More importantly, further studies are required to assess the biological significance of the interaction between DIHD2 and DIERF1.

In summary, the longan fruit DIHD2 and DIERF genes were isolated and characterized. Moreover, DIHD2 and DIERF genes were differentially expressed during longan fruit senescence. Protein–protein interaction analysis indicated that DIHD2 physically interacted with DIERF1, suggesting that they may act together to regulate gene expression involved in fruit senescence. To the best of our knowledge, this is the first report on the interaction between a HD2-type HDAC and an ERF TF. These results suggested the involvement of the epigenetic regulation of gene expression during longan fruit senescence.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Schematic maps of the constructs used in the transcriptional activation analysis in yeast cells.

Figure S2. Schematic maps of the constructs used in the subcellular localization analysis.

Figure S3. Schematic maps of bait and prey constructs used in the yeast two-hybrid assay.

Figure S4. Schematic maps of the constructs for the BiFC assay.

Figure S5. Amino acid sequence alignment of the DIHD2 protein with other plant HD2 proteins.

Figure S6. Phylogenetic tree of the deduced amino acid sequences of DIHD2 and other plant HD2s.

Figure S7. Amino acid alignment of the AP2/ERF domain of DIERFs and other ERF proteins.

Figure S8. Phylogenetic analysis of DIERFs with other AP2/ERF proteins.

Figure S9. Amino acid sequence alignment of Group III ERFs.

Figure S10. Amino acid sequence alignment of Group IV ERFs.

Figure S11. Subcellular localization of DIHD2 in Arabidopsis mesophyll protoplasts.

Figure S12. Relative quantification of DIHD2 (A), DIERF1 (B), and DIERF2 (C) in aril tissues of control and NO-treated longan fruit stored at room temperature.
Figure S13. Relative quantification of DiHD2 (A), DiERF1 (B), and DiERF2 (C) in aril tissues of longan fruit stored at low temperature.

Figure S14. Relative quantification of DiHD2 (A), DiERF1 (B), and DiERF2 (C) in aril tissues of longan fruit stored for 40 d at 4 °C and then transferred to 25 °C.

Figure S15. Western blot analysis of histone H3 acetylation levels in aril tissues of control and NO-treated longan fruit stored at room temperature (25 °C) for 5 d.

Figure S16. BiFC visualization of the DiHD2 and DiERF1 interaction in transiently co-expressed Arabidopsis mesophyll protoplasts.

(see Table S1. Primer sequences used for cloning DiHD2, DiERF1, and DiERF2.

(see Table S2. Primer sequences used for subcloning into pGBK-T7.

(see Table S3. Primer sequences used for fusing GFP.

(see Table S4. Primer sequences used for synthesis of DIG-labelled probes for northern blotting.

(see Table S5. Primer sequences used for yeast two-hybrid and BiFC assays.

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