Expression analysis of NAC genes during the growth and ripening of apples

Qijing Zhang¹,², Tong Li¹, Lijie Zhang¹, Wenxuan Dong¹*, Aide Wang¹

¹Shenyang Agricultural University, Shenyang, China
²Liaoning Institute of Pomology, Yingkou, China

*Corresponding author: wxdong63@126.com

Abstract


Plant-specific NAC transcription factors (TFs) play crucial roles in various pathways related to the stress response. However, to date, little information regarding NAC gene regulation during fruit ripening is available for the apple (Malus domestica). Here, we report that 13 out of 182 MdNAC genes were differentially expressed during the stages of fruit growth and ripening. Sequence analysis indicates that these 13 MdNAC genes harbour distinct structures and potentially diverse functions. The expression of both MdNAC1a and MdNAC78 was repressed by ethylene and induced by 1-MCP during storage. MdNAC2, MdNAC26, MdNAC41, MdNAC57, MdNAC80, MdNAC91, MdNAC119 and MdNAC141 were up-regulated by ethylene and their transcription mirrored ethylene production rates during storage. MdNAC1, MdNAC16 and MdNAC32 did not respond to 1-MCP exposure. Additionally, the 13 MdNAC genes identified displayed differential tissue-specific expression patterns. These results suggest that NAC TFs play an important role in the regulation of apple development via both ethylene-dependent and -independent mechanisms.

Keywords: Malus domestica; NAC TFs; ethylene; fruit development; storage

Apple (Malus domestica), a typical climacteric fruit, is one of the most economically important woody plants in the temperate regions. In climacteric fruits, ethylene synthesis, which increases at the onset of ripening, is the key driver of fruit softening (Bapat et al. 2010). Typically, 1-Methylcyclopropene (1-MCP) is known to inhibit ethylene-induced effects in the tomato, banana, plum, apple and numerous ornamental plants (Sisler et al. 1999). It acts by binding irreversibly to ethylene receptors. Genes associated with ethylene biosynthesis and perception pathways have been identified in the apple, including those encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, ethylene receptors, CTR1 orthologue, ethylene insensitive 3-like (EIL) genes and the ethylene responsive factor (ERF) (Wang et al. 2012). However, information on how the ripening-related NAC transcription factors (TFs) regulate ethylene-induced ripening in the apple is limited.

The NAC TFs have been shown to regulate a number of biological processes, including development (Hao et al. 2011), senescence (Kou et al. 2012) as well as biotic (Christianson et al. 2010) and abiotic stress responses (Mao et al. 2012). Typically, NAC TFs (NAM, ATAF1/2, and CUC2), contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain involved in transcriptional regulation. Some NAC members function as transcription activators while others act as repressors, depending on environmental signals (Hao et al. 2010).

The NOR (non-ripening) gene in the tomato (Solanum lycopersicum) encodes an NAC-domain TF and likely acts to regulate the expression of other genes responsible for ripening phenotypes,
including ethylene production (Martel et al. 2011). AtNAP, a NAC family TF in Arabidopsis, is required for ethylene stimulation of respiration during fruit senescence (Kou et al. 2012). Additionally, some NAC genes affect others upstream of the ethylene signalling pathway, inducing ethylene synthesis during fruit ripening. For example, SINAC4 can interact with both the RIN and NOR proteins, and regulate ethylene-dependent and -independent processes during tomato ripening (Zhu et al. 2014); SlNAC1 exerts a broad influence over-expression through both ethylene-dependent and abscisic acid-dependent pathways (Ma et al. 2014). MaNAC1/2 interacts with a downstream component of ethylene signalling, and an ethylene-insensitive ethylene insensitive 3 (EIN3)/EIN3-like (EIL) gene, termed MaEIL5, was down-regulated during banana (Musa acuminata) ripening (Shan et al. 2012). Moreover, CsNAC (Citrus sinensis) and PsNAC (Prunus salicina) were also reported to be involved in the ethylene signalling pathway during fruit ripening (Fan et al. 2007; Liu et al. 2009).

Although the functions of NAC TFs have been studied extensively in several plants, the involvement of NAC TFs in relation to fruit growth and ripening in the apple has generally received less attention. After screening 182 genes of the NAC family from the recently released apple genome (Su et al. 2013), we found that 13 MdNAC TFs were differentially expressed during apple growth and ripening. These 13 MdNAC genes were sequenced, and their expression profiles during fruit growth and ripening, in fruits and various other tissues, were analysed using quantitative real-time PCR (qRT-PCR) and semi-quantitative RT-PCR. The obtained results are likely to contribute to elucidating how transcription levels affect MdNAC protein activation domains during the regulation of fruit development and ripening.

**MATERIAL AND METHODS**

**Plant materials and treatments.** Five ‘Golden Delicious’ (GD) apples (except for 15 fruits at the –120 stage) were sampled for analysis of ethylene production and for RNA extraction 120, 90, 60, 30 and 15 days (referred to as –120, –90, –60, –30 and –15 d, respectively) before commercial harvest day (referred to as September 0, 20, 2014). Sixty-five mature GD apples (Brix at 12%–13%) were collected on the day of harvest, 20 of which were exposed to 1 μl/l 1-MCP (Fresh Doctor, China) for 24 h in an airtight container (20 l). Another 20 fruits were dipped in a 1,000 ppm ethephon solution for 15 s to induce the release of ethylene, and then stored for 12 h at 22°C in an airtight container (20 l). The third batch of 25 apples was used as a control. Five apples from each treatment were sampled 5, 10, 20 and 30 d after storage at 22°C to compare ethylene production, fruit firmness and to isolate RNA.

Roots, stems, leaves, petioles, peel, pedicels and leaf buds were sampled on the day of harvest, while the flowers were sampled on May 5, 2014. Each of these tissues was taken from three sampled trees, frozen quickly in liquid nitrogen and stored at –80°C for RNA isolation. The sampled trees were 10 years old, and had been grafted onto rootstocks of Malus baccata planted in the Experimental Orchard of the Liaoning Institute of Pomology (Xiongyue, China).

**Measurement of flesh firmness and ethylene production rates.** Flesh firmness was measured with a portable pressure tester (FT-327; Facchini, Italy). The mean values of fruit firmness and the standard deviations (SD) were calculated from five independent experiments. For ethylene measurements, one intact fruit (except for five fruits at the –120 stage) was enclosed in an airtight container (0.86 l) equipped with septa at 22°C for 1 h and 1 ml head space gas was sampled using a syringe. The ethylene concentration was measured with a gas chromatograph (Agilent 7890A) equipped with a flame ionisation detector. An HP-AL/S column (Cat. No. 19095P-S25, Agilent) was used. The mean values of ethylene production and the standard deviations (SD) were calculated from three biological replicates.

**Semi-quantitative RT-PCR and qRT-PCR analysis.** Total RNA was extracted using the CTAB method, with modifications (Gasic et al. 2004). One microgram of total RNA was used to synthesize first strand cDNA with a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa). The primers were designed using Primer3 (http://frodo.wi.mit.edu/) (Table 1). The semi-quantitative RT-PCR was performed using 8 μl Ex Taq mix (Takara), 1 μl of each primer (10 μM), 1 μl of cDNA (50 ng/1 μl) and 4 μl of distilled water at 94°C for 4 min, followed by 22 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. PCR products were separated using electrophore-
sis on 1% agarose gels stained with ethidium bromide. The qRT-PCR consisted of 7.5 ml of SYBR Green PCR Supermix (Bio-Rad Laboratories), 1 μl of 10 mM stocks of each primer, 1 μl of cDNA and distilled water to a final volume of 15 μl under the following conditions: 95°C for 3 minutes, followed by 40 cycles of 10 s at 95°C, 30 s at 56°C and 30 s at 72°C. The apple Actin gene (EB136338) was used as an internal control. The relative expression levels of the target genes were calculated using the Livak method (Livak, Schmittgen 2001). Each qRT-PCR analysis was performed in triplicate.

**Sequence structure analysis.** Analysis of nucleotide sequences was performed using the NCBI Blast program (http://www.ncbi.nlm.nih.gov/Blast; Altschul et al. 1997). The bioinformatics tools available at http://www.expasy.org were used to analyse the identified proteins. Sequence alignments were made using the DNAMAN program, version 5.2.2. ATAF1 (NP_680161.1) and CUC2 (NP_200206.1) from Arabidopsis (Aida et al. 1997; Souer et al. 1996) were included in the analysis.

**Statistical analysis.** Analysis of variance was calculated using the SPSS statistical software package (IBM, Armonk, USA). Comparisons of means was performed using Duncan’s test at $P = 0.05$. For all analyses, a probability value $< 0.05$ was used to determine significance. Error bars indicate standard deviations (SD).

### RESULTS AND DISCUSSION

**Gene structure and protein motif analysis of the 13 NAC TFS**

The 13 MdNAC proteins ranged from 204 aa (MdNAC78) to 607 aa (MdNAC2) in size, with molecular weights of between 23.3 kDa (MdNAC78) and 67.8 kDa (MdNAC2), and pI (isoelectric point) values ranging between 4.64 (MdNAC2) and 8.04 (MdNAC16), respectively (Table 2). Alignment of these full-length proteins clearly revealed that they contained the conserved NAC domain in the N-terminus, which was divided into five subdomains (A–E) (Fig. 1). A putative nuclear localisation signal (NLS) was detected in the C and D subdomains (Kikuchi et al. 2000). The pairwise identity among the aa sequences of the 13 NACs ranged from 10.42% (MdNAC91 and MdNAC119) to 91.14% (MdNAC1 and MdNAC2) and no significant similarities were found among the C-terminal regions. Generally, sequence analysis indicated that the 13 NAC apple gene products harbour distinct structures and potentially have diverse functions.

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Table 1. Primer pairs used in quantitative real-time PCR and semi-quantitative RT-PCR for analysis of the expression profiles of 13 MdNAC genes in response to ethephon and 1-MCP treatments and expression patterns in the eight tissues tested

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
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</thead>
<tbody>
<tr>
<td>MdNAC1a</td>
<td>ACCATTACACACTTTGGCGGG</td>
<td>ATCAAAAGGTTGGACCCCATAATT</td>
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<td>MdNAC1</td>
<td>TTCGACTGGTGCAAGATGAT</td>
<td>GCACTTCCCTCCCTACGAGCAG</td>
</tr>
<tr>
<td>MdNAC2</td>
<td>TGTTTCAGCTGTCACAGATGAC</td>
<td>CATGCTTCTCCCTAATCCTCAA</td>
</tr>
<tr>
<td>MdNAC16</td>
<td>GGCACCTCTTTGCAAGACCTTT</td>
<td>CATACCACTTGGCTCTTTTT</td>
</tr>
<tr>
<td>MdNAC26</td>
<td>TGTCAGGTGTCGTTGTATCTGGG</td>
<td>GGAATGTGATTTCTCTGTAAG</td>
</tr>
<tr>
<td>MdNAC32</td>
<td>CAGTTGGCTTTACAGCTGAGA</td>
<td>CGCAGCTCTTTGGCTCTCAG</td>
</tr>
<tr>
<td>MdNAC41</td>
<td>TGGCAGTTCACTTCCCTACAGGA</td>
<td>ACAATCCCTTTGTGTTGTGAC</td>
</tr>
<tr>
<td>MdNAC57</td>
<td>CTTCCGACCTCCCATTACG</td>
<td>TTGGACATTATGAGCAGAG</td>
</tr>
<tr>
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<td>GGTCACGAGAAATGCGTACTGGA</td>
<td>TGGACTCTCTGTTGAGTCTCT</td>
</tr>
<tr>
<td>MdNAC80</td>
<td>CAGCAAACACACACACACAC</td>
<td>CACGCTCCGTTGAGAAG</td>
</tr>
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<td>MdNAC91</td>
<td>AGAAGGTCAGGGTTGTGAGT</td>
<td>GGGCTTCTCTATTATCGCCC</td>
</tr>
<tr>
<td>MdNAC119</td>
<td>ATGCCGAATGTTAAAGAGAGGT</td>
<td>TCTTTAACAGTGGCAAGAC</td>
</tr>
<tr>
<td>MdNAC141</td>
<td>TGTTTCGTCCTACATTGGCA</td>
<td>AATCTTGTCTTTATGGCACA</td>
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</table>
Fruit firmness and ethylene production

Fruit firmness in the untreated control decreased rapidly after commercial harvest, while fruit treated with 1-MCP maintained high firmness values even after fruits were maintained at room temperature for 30 days (Fig. 2a). However, ethephon treatment caused a more dramatic and rapid reduction in fruit firmness. Accordingly, the ethylene production of untreated apples increased significantly during storage at room temperature, and a peak of ethylene production was observed at 20 days. We found that the 1-MCP treatment significantly blocked ethylene production, which was approximately half that measured in untreated fruit. However, ethephon treatment notably enhanced ethylene production (Fig. 2b), and, therefore, fruit softening was prevented or delayed by 1-MCP, underlining the fact that the effects of treatments are often closely associated with ethylene production (Toivonen, Lu 2005).

Expression profiles of selected MdNAC genes in different tissues

Increasing evidence suggests that tissue-specific expression of NAC TFs plays a critical role in plant growth and development (Berger et al. 2009). MdNAC119 was highly expressed in flowers and old leaves (Fig. 3); thus, it may be related to flower formation and development as well as regulation of leaf senescence. Additionally, MdNAC78 and MdNAC141 were expressed in peels and roots, respectively, suggesting that they may have an important role in both fruits and roots. MdNAC1a was mainly expressed in peels, while MdNAC32 and MdNAC80 were mainly expressed in peels and pedicels, suggesting that the dominant function of these genes is in the fruit portion of the plant. It is interesting to note that 11 of the MdNAC genes analysed (all except for MdNAC119 and MdNAC78) were expressed in fruit and roots, suggesting they might play a role in improving apple stress tolerance, since a report has shown that controlling drought resistance involves regulating root growth and development (Sharp et al. 2004).

Expression profiles of selected MdNAC genes at different fruit growth stages

Gene expression patterns can provide important clues that aid in the determination of gene function (Su et al. 2013). In this study, MdNAC2, MdNAC26, MdNAC80, MdNAC91, MdNAC91 and MdNAC141 genes were expressed higher at –120 d, –60 d, and –30 d and lower at –90 d and –15 d

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Genomic location</th>
<th>Protein accession No.</th>
<th>Length (aa)</th>
<th>Molecular weight (kDa)</th>
<th>Isoelectric point</th>
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</tr>
<tr>
<td>MdNAC2</td>
<td>MDP0000621646</td>
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<td>ADL36811.1</td>
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<td>67.8</td>
<td>4.64</td>
</tr>
<tr>
<td>MdNAC16</td>
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<td>LG2</td>
<td>XP_008354424.1</td>
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<tr>
<td>MdNAC26</td>
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<td>LG3</td>
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<tr>
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<tr>
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<td>MDP0000802924</td>
<td>LG4</td>
<td>XP_008355629.1</td>
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<td>8.00</td>
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<td>MdNAC57</td>
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<td>MdNAC78</td>
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<tr>
<td>MdNAC80</td>
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<td>349</td>
<td>30.0</td>
<td>5.14</td>
</tr>
</tbody>
</table>

protein accession numbers from Genbank; gene ID, linkage group, length, molecular weight and isoelectric point as described in Su et al. (2013)
Fig. 1. Multiple sequence alignment of 13 apple MdNAC proteins and two Arabidopsis NAC proteins (ATAF1 and CUC2). Identical amino acids are shaded in black, and similar amino acids are shaded in grey. The locations of the five highly conserved subdomains (A–E) are indicated above the sequences and the degenerate bipartite nuclear localisation signal (NLS) is indicated with an arrow.
during fruit development, which follows the ethylene production pattern (Fig. 2b). The differential expression of *MdNACs* might regulate the expression of ethylene-associated genes in the vegetative developmental stage of the apple. Additionally, *MdNAC1a, MdNAC2, MdNAC16, MdNAC41, MdNAC57, MdNAC91* and *MdNAC141* were most highly expressed during young fruit development (~120 d), with levels significantly higher than at later growth stages (Fig. 4). During this early stage of development, fruit tissue undergoes several rounds of cell division followed by cell expansion (Jansen et al. 2008), suggesting that the NAC proteins might be involved in fruit cell differentiation.
Fig. 4. Transcriptional analysis of the 13 *MdNAC* genes

GD denotes non-treated Golden Delicious apple fruits, GD 1-MCP indicates GD treated with 1-MCP and GD Eth denotes GD treated with ethephon.

different letters indicate significant differences among GD fruits during storage; *statistically significant differences between GD, 1-MCP and Eth fruits
Fig. 4 to be continued Transcriptional analysis of the 13 MdNAC genes

GD denotes non-treated Golden Delicious apple fruits, GD 1-MCP indicates GD treated with 1-MCP and GD Eth denotes GD treated with ethephon

Different letters indicate significant differences among GD fruits during storage; *statistically significant differences between GD, 1-MCP and Eth fruits
Expression profiles of selectedMdNAC genes in treated and untreated fruit

It has been reported that AtNAC2 (Arabidopsis), a NAC-type TF gene induced by the ethylene precursor 1-aminoacyclopropane-1-carboxylic acid (ACC), may be a common downstream component of the ethylene signalling pathway (He et al. 2005). During fruit ripening stages, the expression of MdNAC2, MdNAC26, MdNAC41, MdNAC57, MdNAC80, MdNAC91, MdNAC119 and MdNAC141 was induced by exogenous ethylene and inhibited by 1-MCP treatment, and the transcription of these genes mirrored ethylene production rates. The expression of MdNAC78 and MdNAC1a, meanwhile, was inhibited by exogenous ethylene and induced by 1-MCP (Fig. 4). This suggests that MdNAC genes may be involved in apple fruit ripening via interactions with ethylene signalling components. However, MdNAC1, MdNAC16 and MdNAC32 did not respond to 1-MCP, even though they exhibited differential expression during growth and storage, suggesting that they might regulate other genes involved in ripening or fruit development independently of ethylene. It was previously found that MdNAC41, MdNAC57, MdNAC80 and MdNAC119 responded to one or more of four abiotic stress treatments (low temperature, drought, high salinity and exogenous abscisic acid (ABA)) of apple rootstock (M. Hupehensis) (Su et al. 2013); in our study, these genes also responded to ethylene and 1-MCP treatments, further indicating that MdNAC TFs play multiple roles in apples.

In this study, we presented an analysis of the NAC gene family during apple fruit development and ripening. The results will help lay the foundation for the functional characterisation of the NAC gene family and may facilitate an understanding of the molecular basis of the regulatory network involved in the ripening of apple fruit.

References


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