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

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Abstract

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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,

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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, SlNAC4 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 on tomato ripening, in part by regulating SlNAC1 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-

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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

Primer	Forward (5'-3')	Reverse (5'-3')
MdNAC1a	ACCATTACACACTTTGCCGGG	ATCAAACGGTGGACCCATACATT
MdNAC1	TTCAGCTGTCGCAGAATGAT	GCATCTTCCCCTAGTCAGCAG
MdNAC2	TGTTCAGCTGTCACAGGATGAC	CATGCTTCTTCCCCTAATCACTAA
MdNAC16	GGCACTTCTTCCATAGACCTT	CATCACCCAATTCGTCTTTT
MdNAC26	TGTCGAGTGTACGTTGTATCTGGG	GGAATGTGAATAAGTTTCGTCTGA
MdNAC32	CGGTTGCTATTATCGCTGAA	CTGCCCTATTGGGTCTCG
MdNAC41	TTGCAGTACTTCCCTTCACGAGA	ACATATCCTCTTGCTTGGTGTGAC
MdNAC57	CTTCCGAGTCCCATTACG	TTGGAGCATTATGAGCAGAG
MdNAC78	GGGTCACGAATAATGGGTACTGGA	TGATGCCTGAATTTGGAGCTTCT
MdNAC80	CAGCAACAACCACCACAAC	CCAGCGTCCTGTGAGAAC
MdNAC91	AGAAGGCAGGTCTTGTGGAGT	GGGCTTTCTCTATTATCGCCCC
MdNAC119	ATGCCGAATTATAAACGAGGAGGT	TCTTTAACAGTGCCGGCAGAC
MdNAC141	TGTTCGTCACATTGGAATTCAGCA	ACATCTCGTTCTTCCTAGGCACA

an "a" was added to MdNAC1a (Su et al. 2013) to differentiate it from MdNAC1 (Wang et al. 2012)

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 2. Basic information regarding the 13 *MdNAC* genes

Gene name	Gene ID	Genomic location	Protein accession No.	Length (aa)	Molecular weight (kDa)	Isoelectric point
MdNAC1a	MDP0000882983	LG1	XP_008359582.1	393	44.5	4.85
MdNAC1	MDP0000457996	LG16	XP_008339779.1	602	67.4	4.65
MdNAC2	MDP0000621646	LG16	ADL36811.1	607	67.8	4.64
MdNAC16	MDP0000124509	LG2	XP_008354424.1	344	38.3	8.04
MdNAC26	MDP0000130797	LG3	XP_017183865.1	242	27.3	6.36
MdNAC32	MDP0000868419	LG3	NP_001280984.1	364	40.7	7.77
MdNAC41	MDP0000802924	LG4	XP_008355629.1	354	40.3	8.00
MdNAC57	MDP0000911724	LG6	XP_008374563.1	361	40.7	7.66
MdNAC78	MDP0000240094	LG8	ADL36791.1	204	23.3	4.95
MdNAC80	MDP0000276765	LG9	ADL36806.1	350	39.8	6.32
MdNAC91	MDP0000309351	LG10	XP_008383681.1	324	36.3	7.76
MdNAC119	MDP0000655623	LG13	XP_008389427.1	236	26.7	4.86
MdNAC141	MDP0000152774	LG15	XP_008337056.1	349	30.0	5.14

protein accession numbers from Genbank; gene ID, linkage group, length, molecular weight and isoelectric point as described in Su et al. (2013)

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

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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

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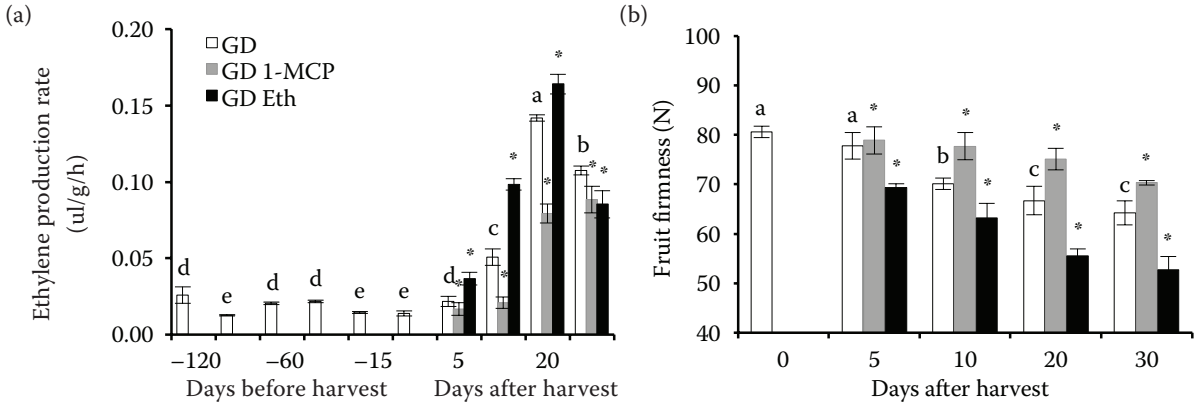

Fig. 2. Changes in fruit firmness and ethylene production

GD – non-treated 'Golden Delicious' (GD) apple fruits, GD 1-MCP – 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; mean values of fruit firmness and the standard deviations (SD) were calculated from five independent experiments; mean values of ethylene production and the standard deviations (SD) were calculated from three biological replicates

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, *MdNAC1*a, *MdNAC2, MdNAC16*, *MdNAC*41, *MdNAC*57, *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 (Janssen et al. 2008), suggesting that the NAC proteins might be involved in fruit cell differentiation.

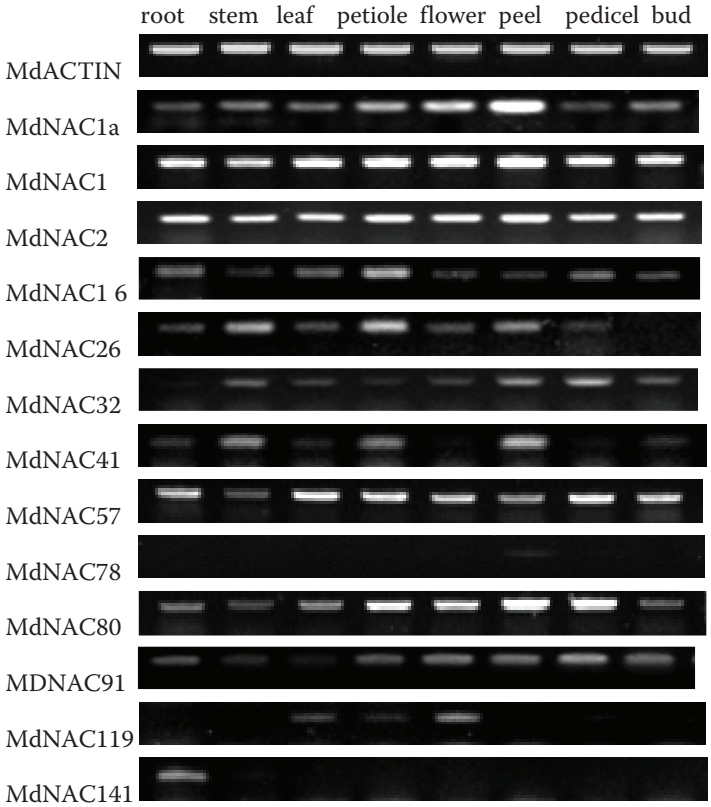

Fig. 3. Expression profiles of 13 MdNAC genes in the different tissues

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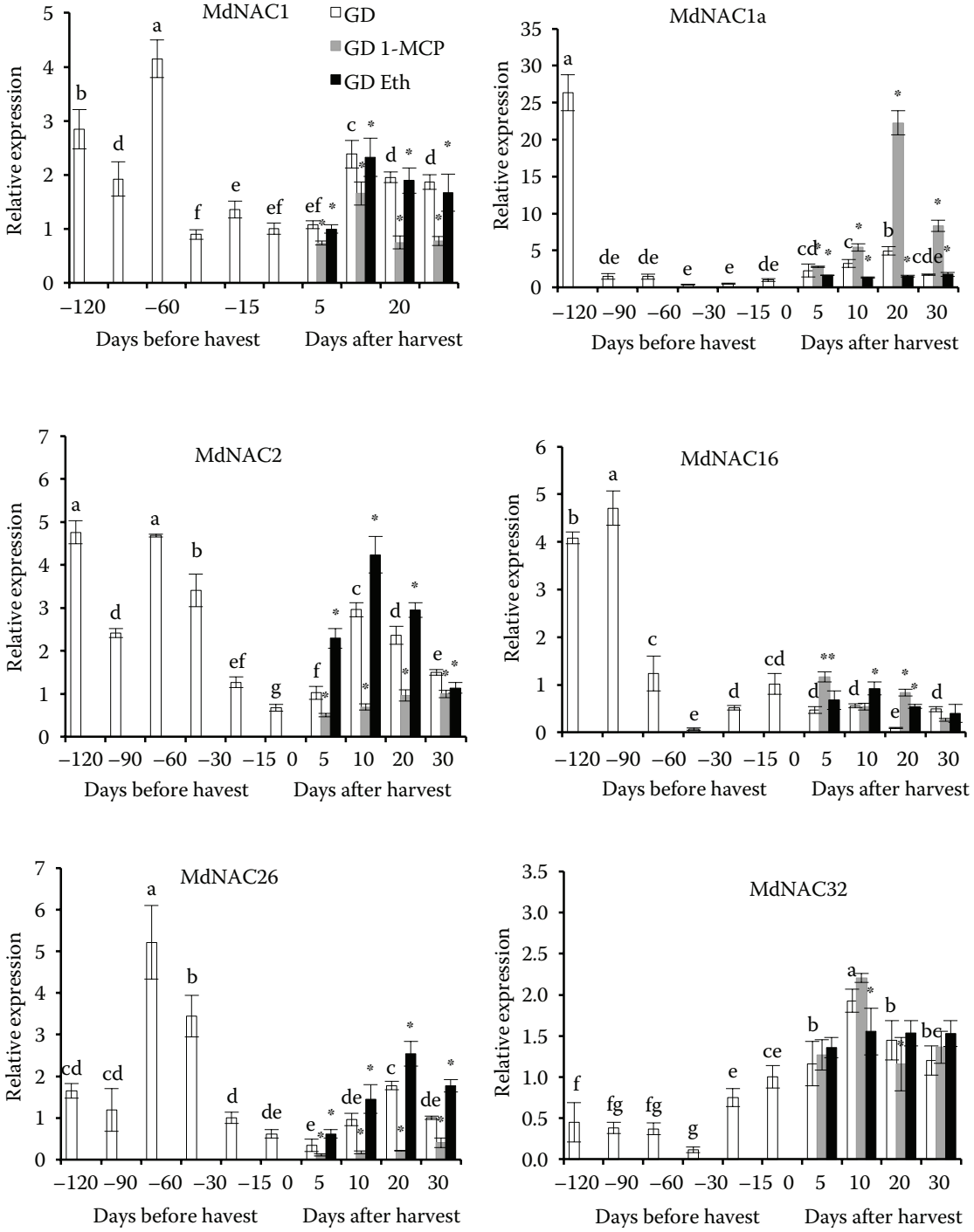

Fig. 4. Transcriptional analysis of the 13 *MdNAC* genes

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

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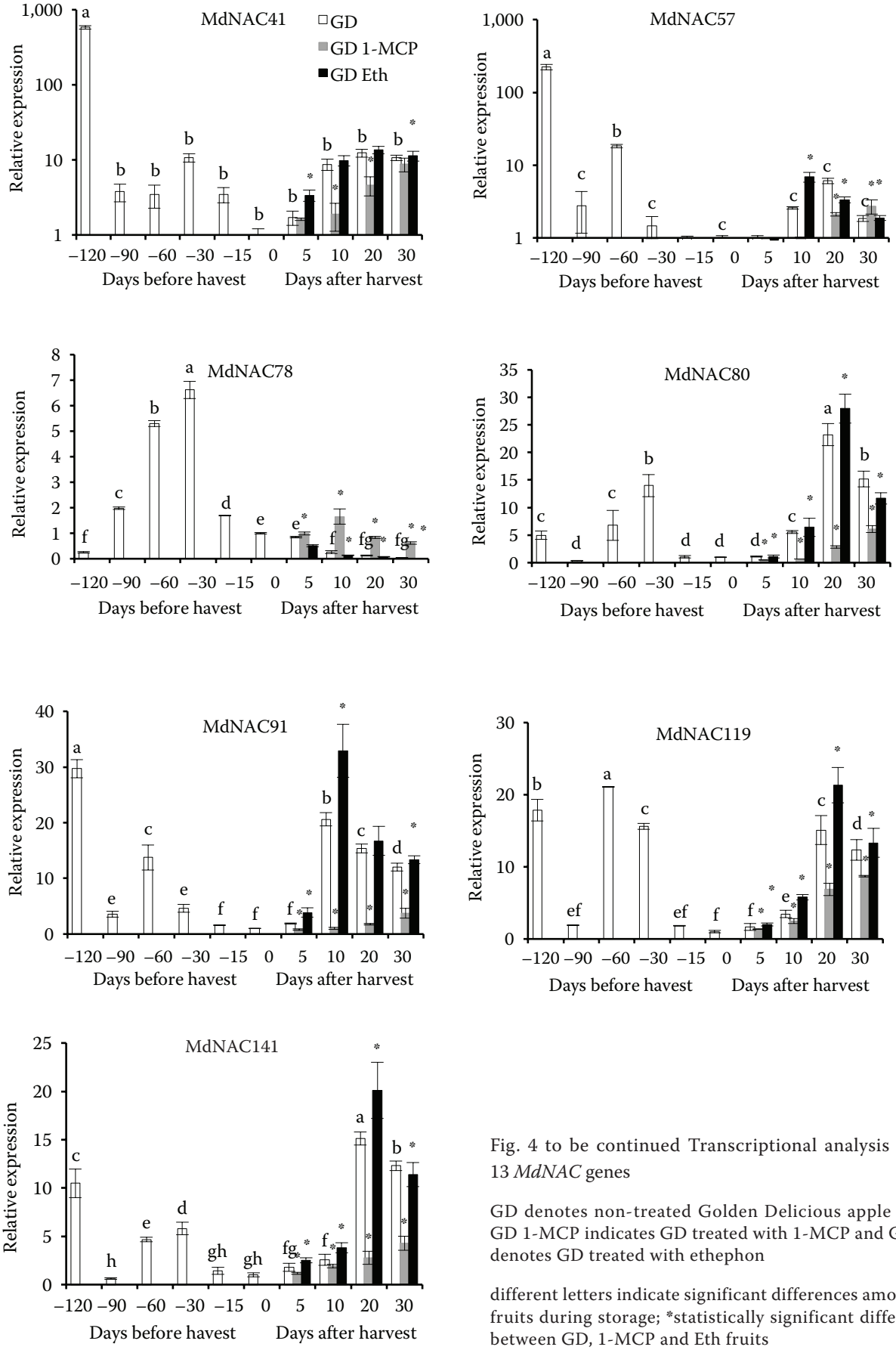

Fig. 4 to be continued Transcriptional analysis of the 13 *MdNAC* genes

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

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Expression profiles of selected *MdNAC* genes in treated and untreated fruit

It has been reported that *AtNAC2* (*Arab*idopsis), a NAC-type TF gene induced by the ethylene precursor 1-aminocyclopropane-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.

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