The Effect of 1-MCP on the Expression of Several Ripening-related Genes in Strawberries

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Abstract. To elucidate the role of ethylene in nonclimacteric fruit development and ripening, quantitative (cDNA–amplified fragment length polymorphism) cDNA–AFLP was used to visualize differential gene expression in four stages of ripening of strawberries (*Fragaria × ananassa* Duch. ‘Elsanta’) treated with 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action. The proportion of clones affected by 1-MCP treatment was much higher in green than in white, pink, and red receptacle tissue. Three major cell-wall-related genes were affected by 1-MCP and, thus, are putatively ethylene dependent: a ripening-repressed beta-galactosidase (Faβgal3), up-regulated by 1-MCP; a putative endo-1,3-1,4-beta-D-glucanase (EGase), up-regulated in green and down-regulated in red fruit by 1-MCP; and a pectate lyase B (plB), expressed only in the red stage and significantly down-regulated by 1-MCP. Furthermore, we have identified genes encoding an alcohol dehydrogenase, a protein kinase-related protein, and a putative glutathione S-transferase, all ripening-induced and down-regulated by 1-MCP, suggesting that their regulation is at least partly ethylene dependent.

Strawberries (*Fragaria × ananassa* Duch.) are considered nonclimacteric, since they do not exhibit a peak in respiration and ethylene production during ripening. Ethylene is present in strawberries, but its influence on fruit ripening is not fully understood. Strawberries can ripen without exogenous ethylene treatment, but exogenous ethylene induces the acceleration of fruit color development and softening (Tian et al., 2000; Wills and Kim, 1995). The levels of ethylene in strawberries decrease from the green to white stage and then rise again slightly in the red-ripe stage (Perkins-Veazie et al., 1996). Ethylene may also play a role in ripening of other nonclimacteric fruit like orange and pineapple (Goldschmidt et al., 1993; Alonso et al., 1995; Cazzonelli et al., 1998). Ethylene induces enhanced anthocyanin accumulation in grapes (El-Kereamy et al., 2003), and a ripening-induced alcohol dehydrogenase gene shows ethylene regulation (Tesniere et al., 2004), suggesting that an ethylene signal transduction pathway could be involved in the control of nonclimacteric ripening.

1-Methylcyclopropene (1-MCP) inhibits ethylene action by binding to ethylene receptors so that ethylene cannot bind and elicit response,

permitting in this way to elucidate the effect of both endogenous and exogenous ethylene (Sisler et al., 1996). 1-MCP, in addition to research purposes, is widely used on ornamental and edible crops to extend their postharvest life (Blankenship and Dole, 2003). In strawberries, the effect of 1-MCP depended mainly on the concentration and the temperature of the postharvest storage conditions. Fumigation with 1-MCP at 5 to 15 nL·L⁻¹ also extended the postharvest life by 35% at 20 °C and 150% at 5 °C (Ku et al., 1999), but at a higher 1-MCP concentration (500 nL·L⁻¹), there was an accelerated loss of quality with a significant decrease in postharvest life at both 20 and 5 °C (Ku et al., 1999). Jiang et al. (2001) also described that lower 1-MCP concentrations (10 to 200 nL·L⁻¹) inhibited ethylene production and maintained fruit firmness and color, but postharvest life was negatively influenced by high (500 to 1000 nL·L⁻¹) 1-MCP concentrations, especially due to the decreased disease resistance since high 1-MCP concentrations blocked phenylalanine ammonia-lyase (PAL) activity, and lowered increases in anthocyanin and phenolic contents. This agrees with the earlier observation of Jersch et al. (1989), that phenolics and elevated activity of PAL were associated with resistance in strawberries to pathogens.

Ripening-induced and ethylene-regulated genes and the effect of different ripening inhibitors have mostly been studied in climacteric fruit. In the present study we report the identification of genes potentially regulated by ethylene in nonclimacteric strawberries by using a cDNA–amplified fragment length polymorphism (cDNA–AFLP) method in 1-MCP treated fruit. The method used is an improved version of cDNA–AFLP and was developed

to allow global, quantitative gene expression analysis (Breyne et al., 2003).

Materials and Methods

‘Elsanta’ strawberries were harvested from a commercial farm near Ghent, Belgium. In total, 5 to 10 green, white, pink, and fully ripe red fruit (without removing the calyxes) were treated with 1-MCP (15 nL × L⁻¹) in 5-L sealed jars for 10 h at 22 °C. 1-MCP was released from SmartFresh powder (Rohm and Haas, Philadelphia, Pa.). Control fruit were kept in the same conditions except for 1-MCP treatment.

cDNA–AFLP experiment. Total RNA was extracted from the fruit flesh according to Salzman et al. (1999). For RNA extractions minimum five fruit per ripening stage were used. First-strand cDNA synthesis was carried out starting from 10 µg of total RNA, and quantitative cDNA–AFLP analysis was conducted as described by Breyne et al. (2003). Restriction enzymes BstYI and MseI (New England Biolabs, Beverly, Mass.) were used, and for preamplifications an MseI primer without selective nucleotides was applied in pair with a BstYI primer containing a C as a selective nucleotide. (The primer sequences were 5'-GACTGCGTAGTGATCC-3' for BstYI and 5'-GATGAGTCCTGAGTAA-3' for MseI). In the cDNA–AFLP reactions 48 primer combinations were tested, both primers contained two selective nucleotides. Amplification products were separated on 5% polyacrylamide gels using the SequiGen system (Bio-Rad, Hercules, Calif.). Dried gels were exposed to BioMax films (Kodak) and scanned with a PhosphorImager 445 SI (Amersham Biosciences, Little Chalfont, U.K.).

Characterization of AFLP fragments and quantitative measurement of the expression profiles. Based on the autoradiogram, the TDFs (transcript derived fragments) were excised from gels and hydrated in 100 µL water for 1 h before PCR amplification of 2 µL by using the same primers as in the selective amplification. Sequence information was obtained either by direct sequencing of the re-amplified product with the BstYI primer or after cloning the fragments in pGEM-T Easy (Promega, Madison, Calif.). The reactions were analyzed with an ABI Prism 310 Genetic Analyzer. Nucleotide and translated amino acid sequences were analyzed for homology to known gene sequences using the BLASTN and BLASTX programs. Quantitative measurement of the expression profiles was done as described by Breyne et al. (2003). Scanned gel images were quantitatively analyzed with the AFLP QuantarPro image analysis software (Keygene N. V., Wageningen, The Netherlands) by which all visible AFLP fragments were scored and individual band intensities were measured in each lane. The raw data were corrected for differences by using a total lane intensity correction. The intensity values were summed per lane for each primer combination and each of the sums was divided by the maximal summed value to yield the correction factors. Finally, all raw data were divided by these correction factors. For the corrected data, the coefficient of

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variation (CV) ($CV = \text{standard deviation}/\text{mean}$) was calculated for each TDF, and a threshold of 2 was chosen for significantly differentially expressed genes.

Results and Discussion

From 86 green receptacle specific clones, 43 were down-regulated by 1-MCP, 33 were up-regulated, and 10 clones showed no change in expression pattern after the treatment. Among the ripening induced clones a much lower percentage was regulated by 1-MCP. From 84 ripening specific TDFs, 20 were down-regulated, 9 were up-regulated, the expression pattern of the rest was not effected by 1-MCP. The difference in response to 1-MCP of the green (88%) and ripening fruit (34%) might be due to their different sensitivity to ethylene, the consequence of the reduced endogenous ethylene production in the ripe fruit compared with the green receptacle. We focused on one ripening repressed and several ripening induced genes with modified expression pattern in response to 1-MCP treatment. Fruit soften-

ing, especially in strawberries, is an important postharvest quality trait, thus one of our main interest was to determine the effect of 1-MCP on genes related to cell-wall metabolism. We identified 3 cDNA-AFLP clones encoding enzymes responsible for cell wall loosening during cell expansion and fruit growth or ripening. One of them—C13M214M008—is ripening repressed, expressed only in the green receptacle, shows homology to beta-galactosidase (Fa β gal3) from strawberries (AJ278705), a cell wall hydrolase, mediating reversible wall loosening and thus allowing turgor-driven cell growth and expansion during fruit growth beside other cell wall modifying proteins. Trainotti et al. (2001) reported, that the transcript amount of this gene appears to be very high in flowers and in young fruits (both small and large green ones), then it gradually decreases during ripening and becomes almost undetectable in red fruits. In the experiment described by Trainotti et al. (2001), the exposure to ethylene of small green fruits and continuance of growth in the presence of ethylene did not seem to particularly affect

the expression of Fa β gal3. In contrary, in our case, Fa β gal3 is significantly up regulated by 1-MCP (Fig. 1A).

The other clone (C23M21M011) accounts for a putative protein similar to endo-1,3-1,4-beta-D-glucanase from rice (AAU10802). It is present in the green receptacle, where it is up regulated by 1-MCP, and in ripe fruit, down regulated by 1-MCP. Though the cDNA-AFLP fragment with homology to endo-1,3-1,4-beta-D-glucanase shows no homology to any of the previously described EGase strawberry genes (Trainotti et al., 1999; Llop-Tous et al., 1999). To prove that we have identified a new member of the EGase gene family, the isolation of the full-length cDNA is in progress. The two, already described, EGase genes, FaEG1 and FaEG3, are predominantly expressed during the ripening process, in the case of FaEG3, the gene expression was also observed in large green fruit and, at low levels, in young vegetative green tissues (Spolaore et al., 2003; Trainotti et al., 1999). There are no data about the ethylene regulation of these genes in strawberry. The sequence analysis of the FaEG1 and FaEG3

Fig. 1. Quantitative expression profile of genes encoding beta-galactosidase (A) and pectate lyase (B) in green, white, pink, and red fruit based on the relative, variance-normalized cDNA-AFLP expression data. Gray bar = control fruit, black bar = 1-MCP treated fruit.

Fig. 2. Quantitative expression profile of genes encoding alcohol dehydrogenase (A), protein kinase-related (B), and putative glutathione S-transferase (C) in green, white, pink, and red fruit based on the relative, variance-normalized cDNA-AFLP expression data. Gray bar = control fruit, black bar = 1-MCP treated fruit.

promoters revealed the presence of ethylene responsive element, ATTTCAAA, identified in the promoter region of an ethylene responsive glutathione-S-transferase gene from *Dianthus caryophyllus* (Itzhaki et al., 1994) suggesting that these genes might be ethylene regulated. The fact that in our experiment 1-MCP affects the transcript level of endo-1,3-1,4-beta-D-glucanase sustains this possibility.

The third clone (C14M31M010) is ripening specific, encodes a pectate lyase B already identified in strawberry (AF339024) (Benitez-Burraco et al., 2003). Pectate lyases randomly cleave β -1,4 linked galacturonosyl residues of pectines from the middle lamella and primary cell-walls of higher plants, resulting in the maceration of plant tissues (Collmer and Keen, 1986). It was already reported that the expression level of all members of the pectate-lyase gene family were strongly decreased when the red fruit were kept in controlled atmosphere enriched with CO₂ (Benitez-Burraco et al., 2003). The same pattern was observed by us after 1-MCP treatment, which dramatically decreases the gene expression level of pectate lyase B (Fig. 1B). Furthermore a ripening-related alcohol dehydrogenase homolog to tomato (S39508) was identified (C13M214M007). Transcript accumulation is induced during the onset of ripening, and 1-MCP partly inhibits the gene expression (Fig. 2A). Similar results were reported in a recent study, showing that the induction of VvADH2 transcription, both in grapevine berries at the inception of ripening and in cell suspensions, was reduced by 1-MCP. The same authors show that treating cell suspensions with 2-chloroethylphosphonic acid (2-CEPA), an ethylene-releasing compound, also resulted in a significant increase of ADH activity and VvADH2 transcription (Tesniere et al., 2004). These data suggest that ethylene could be involved in the control of ADH expression in nonclimacteric fruits. Another cDNA-AFLP fragment is similar to a gene encoding a putative glutathione S-transferase from tobacco (Q03666). It exposes a ripening induced pattern, while 1-MCP down regulates its transcript level in the pink and red stages (Fig. 2B). Strawberry glutathione S-transferases have already been identified as ripening regulated and oxidative stress induced (Aharoni et al., 2002) but there is no data about the involvement of ethylene in its regulation in this species. Ethylene regulation of several members of glutathione S-transferase gene family was reported in carnation (Itzhaki et al., 1994) and *Arabidopsis* (Zhong and Burns, 2003) during stress response or developmental processes (Mang et al., 2004). One of the most intriguing clones accounts for putative protein kinase from *Arabidopsis* (NP_177209). Its expression level increases during ripening, and is reduced by 1-MCP exposure in pink and red stages (Fig. 2C). Further functional analyses are needed to assess whether this gene could be involved in oxidative stress related signaling

during ripening, and regulated by ethylene.

The genes, reported here, were submitted to NCBI data bank and appeared under the following accession numbers: DQ011162 (alcohol dehydrogenase), DQ022748 (putative endo-1,3-1,4-beta-D-glucanase), AAW82451 (putative glutathione-S-transferase), and AY679612 (putative protein kinase).

In this paper we described several genes identified by cDNA-AFLP, showing altered expression in response to 1-MCP, an ethylene inhibitor, thus gaining insight into some potentially ethylene dependent processes in strawberry ripening. However, the effect of 1-MCP on prolongation of postharvest life of strawberries is contradictory (Bower et al., 2003), we found, that 1-MCP is suitable for scientific studies of nonclimacteric maturation at molecular level.

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