



Comparison of the expression of chlorophyll-degradation-related genes during ripening between stay-green and yellow-pear cultivars



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ABSTRACT

Chlorophyll degradation is a key component of the changes in pigment composition that typically occur in fruit peel at the onset of ripening. In this study, two pear cultivars, which are different in color development patterns, were used to clarify the molecular mechanism of chlorophyll degradation during ripening. Fruit was harvested and stored at 1 °C for cold treatment and then transferred to 20 °C for ripening. 'Le Lectier' pears turned yellow during ripening with concomitant loss of chlorophylls *a* and *b*, and carotenoids. In contrast, 'La France' pears stayed green even when fully ripe. In this cultivar, chlorophyll and carotenoid contents did not change significantly. Ethylene was normally produced in 'La France' pears, indicating that the stay-green characteristics do not result from an inability of the fruit to produce ethylene. The expression levels of the chlorophyll degradation related genes, including *chlorophyllase 1*, *pheophorbide a oxygenase*, *non-yellow coloring 1*, and *NYC1-like* were lower in 'La France' than those in 'Le Lectier' pears. Suppression of these genes in 'La France' pears might be related to the stay-green characteristics when fully ripened.

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1. Introduction

The intense uniform green color of many vegetables is highly attractive. Yellowing and rapid senescence are major post-harvest problems of leafy vegetables, which result from chlorophyll degradation (Siomos and Koukounaras, 2007). In contrast, color development during ripening in most fruit is attributed to the edible phase, particularly in oranges, mangos, persimmons, and bananas. The appearance of pear fruit is also important for consumer acceptance, and is characterized by the pigment of its peel during ripening.

Pears are usually harvested at the mature green stage and ripen at ambient temperature. Green, red, and russet are three fruit color terms that describe the mature green stage of pear fruit. Moreover, the green type is subdivided into two groups. One includes cultivars such as 'Bartlett' pears, in which the peel color turns from green to yellow during ripening. The other includes cultivars such as 'Beurré d'Anjou', in which peel color shows a very subtle change. Chlorophylls and carotenoids are related to the color change from green to yellow. When fruit appears green in bananas, an abundance of

chlorophyll masks the carotenoids. The yellow color of carotenoids is unmasked by chlorophyll degradation during ripening.

Chlorophyll breakdown is an enzymatic process. The early step of chlorophyll degradation is the removal of phytol from chlorophyll *a* and formation of the green product, chlorophyllide by chlorophyllase (CLH) (Harpaz-Saad et al., 2007; Matile et al., 1999). After the Mg atom is removed from the central core of chlorophyllide by Mg dechelataase, pheophorbide *a* is converted to a red chlorophyll catabolite by pheophorbide *a* oxygenase (PAO). This conversion results in opening of the ring structure of the chlorophyll backbone leading to loss of green color (Hörtensteiner, 2006; Suzuki et al., 2006). An alternative pathway for chlorophyll degradation is formation of pheophorbide *a* under the function of pheophytinase (PPH) (Schelbert et al., 2009).

The enzymes that degrade the light harvesting complex (LHC)-chlorophyll complexes, chlorophyll *b* reductase and 7-hydroxymethyl chlorophyll *a* reductase, are involved in the catalysis of chlorophyll *b* and the conversion to chlorophyll *a* (Rudiger, 2002). In addition, non-yellow coloring 1 (NYC1) is required for functioning of the chlorophyll *b* reductase complex (Sato et al., 2009). NYC-like (NOL) is closely related to NYC1, and expression of both genes is enhanced in senescing tissues. The function of the NYC1 and NOL proteins led to the hypothesis that they may directly interact to breakdown chlorophyll *b* by

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chlorophyll *b* reductase. However, recombinant *NYC1* does not have such activity, but the *NOL* recombinant protein converts chlorophyll *b* to 7-hydroxymethyl chlorophyll *a*, the early breakdown product of chlorophyll *b*, indicating that *NOL* interacts with chlorophyll *b* reductase activity in vitro (Kusaba et al., 2007).

Expression of the *stay-green* (*SGR*) gene is induced at the onset of leaf senescence or fruit ripening and is associated with chlorophyll breakdown (Armstead et al., 2006; Park et al., 2007; Sato et al., 2007). However, the specific role of *SGR* in chlorophyll degradation remains unknown. Most reports suggest that *SGR* directly interacts with a subset of proteins contained within the light harvesting chlorophyll *a/b*-protein complex II (Park et al., 2007). This observation suggests that *SGR* might be involved in degrading pigment protein complexes during senescence. Furthermore, the expression level of an *Arabidopsis* *SGR* homolog in the *pao1* mutant is reduced compared with that of the wild-type, leading to a reduction in *SGR* protein levels in the *pao1* mutant (Ren et al., 2007; Park et al., 2007).

'La France' pears are a popular cultivar and account for ~70% of pear production in Japan. This cultivar stays green even at the fully ripe stage. This causes judgment difficulties for the edible phase by consumers. Thus, it is important to clarify the mechanisms underlying stay-green traits. In the present study, we investigated the molecular mechanism of chlorophyll degrading genes using the 'La France' and 'Le Lectier' pear cultivars, which have different patterns of color change. The latter is a local cultivar and develops yellow color during ripening, in contrast to the 'La France' pear.

2. Materials and methods

2.1. Plant material

'La France' and 'Le Lectier' pear fruits were harvested at the mature green stage from a commercial orchard near Yamagata, Japan. The fruit was selected for uniform color, shape, and without damage or visual defects and then exposed to cold storage at 1 °C. After the cold-storage period, all fruits were transferred to 20 °C for ripening for the duration of the experiment. Five fruits were sampled every 3 days after cold storage.

2.2. Measurement of ethylene production and flesh firmness

Individual fruit were placed in 1.5-l glass desiccators, which were flushed with air and then sealed for 1 h. A 1-ml gas sample was withdrawn using a syringe and injected into a gas chromatograph (model GC-8A; Shimadzu Co., Kyoto, Japan) fitted with an activated alumina column and a flame ionization detector. Flesh firmness was determined on the opposite sides of each pear fruit using a rheometer (Sun Scientific, Tokyo, Japan) with an 8-mm plunger.

2.3. Color measurements

Color measurements were performed on the equator of the fruit with a color spectrophotometer (NF333; Nippon Denshoku, Tokyo, Japan). The meter was calibrated using the manufacturer's standard plate. Hunter values (L^* , a^* , b^*) were monitored on two opposite sides of the fruit. Peel discs of each fruit were prepared using a cork borer (20 mm in diameter). Two discs from each fruit were extracted with 80% acetone. Chlorophyll *a*, chlorophyll *b*, and carotenoids were determined according to Arnon's equations (1949). The results are expressed as mg cm^{-2} .

2.4. RNA isolation and quantitative polymerase chain reaction (qRT-PCR)

Total RNA samples were isolated from fruit peel using the RNeasy Mini Kit (Takara Bio, Shiga, Japan). First-strand cDNA was

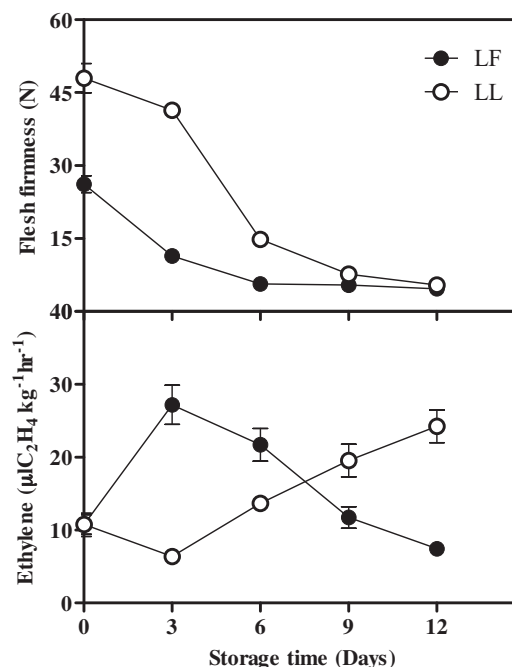


Fig. 1. Changes in ethylene production (A) and flesh firmness (B) in 'La France' and 'Le Lectier' pears during ripening at 20 °C. Values are means \pm standard errors ($n = 5$).

synthesized from total RNA using PrimescriptTM II RTase (Takara Bio), following the manufacturer's instructions. Quantitative PCR mixtures were prepared using the FastStart Essential DNA Green Master Kit (Takara Bio) on a LightCycler Nano System (Roche Diagnostics, Indianapolis, IN, USA). Primer sequences for each gene were designed based on the sequences of *Pyrus bretschneideri* published in the NCBI database (Table 1). *EF1- α* was used as the reference gene. qRT-PCR analysis was performed according to LightCycler Nano software ver. 1.0.3., and the relative expression levels of the target genes were calculated following the $2^{-\Delta\Delta CT}$ formula.

2.5. Statistical analysis

Experiments were performed according to a completely randomized design. All data were analyzed by one-way analysis of variance using the SAS software package (SAS Institute, Cary, NC, USA). Data with a significant *F*-value were subjected to Tukey's test for comparisons.

3. Results

3.1. Changes in ethylene production and flesh firmness during ripening

Cold storage before ripening simulated ethylene biosynthesis, and significant ethylene were produced on day 0 in both cultivars (Fig. 1A). The ethylene production rate of 'La France' pears increased immediately after placing the fruits at 20 °C, peaked on day 3, and then decreased gradually. In contrast, ethylene increased gradually after day 3 in 'Le Lectier' pears.

Flesh firmness of 'La France' pears decreased rapidly immediately after placing the fruits at 20 °C (Fig. 1B). The fruit reached edible firmness and was fully ripened on day 6. Flesh firmness of 'Le Lectier' pears decreased rapidly after day 3, concomitant with the increase in ethylene production.

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