JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Chlorophyll Catabolism Pathway in Fruits of *Capsicum annuum* (L.): Stay-Green versus Red Fruits

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The aim of the present study is to investigate the chlorophyll catabolism pathway of wild-type red and stay-green mutants of *Capiscum annuum* (L.) fruits. In the wild-type red lines chlorophyll catabolism is concomitant with the start of carotenogenesis, whereas in the stay-green mutant lines the chlorophylls coexist with that process, even in over-ripe fruit. During the first stages of ripening, the chlorophyll *a*/chlorophyll *b* ratio is similar for both genotypes, but as ripening proceeds, the ratio in the stay-green lines becomes very high as a result of a blocked degradation of chlorophyll *a* while chlorophyll *b* is degraded at a normal rate. The absence of dephytylated chlorophylls in the wild-type lines distinguishes these from the mutant lines, in which there is a sequential accumulation of chlorophyllide *a* and pheophorbide *a*. Allomerized chlorophylls ($13^2$-OH-chlorophyll *a* and *b*) have also been identified in the catabolic process of the mutant lines, but are absent from the wild type. Consequently, an alteration in pheophorbide *a* oxygenase (PaO) activity seems to be responsible for the stay-green genotype in the lines of pepper analyzed in this study.

**KEYWORDS:** ***Capsicum annuum*** **(L.); chlorophyll catabolism; chlorophyll catabolites fruits; pepper; stay green**

## INTRODUCTION

Chlorophyll catabolism is a very complex process taking place during all of the plant cycle, but it is more active and evident during leaf senescence and fruit ripening. It is generally accepted that the chlorophyll degradation pathway consists of two main stages, the early and the late one, that is, before and after the cleavage of the macrocycle ring. During the early stage there are two main steps involving two different enzymes: chlorophyllase and magnesium dechelatase (*1*). The late stage leads to the cleavage of the tetrapyrrole macrocycle by the action of pheophorbide *a* oxygenase, yielding colorless fluorescent and nonfluorescent catabolites that are further degraded by several reactions. Genes for chlorophyllase and PaO have recently been cloned, which is helping to elucidate the physiological roles of these enzymes (*2*−*4*).

Chlorophyllase, an intrinsic membrane-bound enzyme, catalyzes the first step by hydrolyzing the phytol ester and leading to the formation of chlorophyllide. Magnesium dechelatase is responsible for the removal of $Mg^{2+}$ ions to produce the Mg-free derivatives, pheophytins and pheophorbides, from chlorophylls and chlorophyllide, respectively. The third involved enzyme (PaO) is activated by the ripening and senescence process (senescent-specific fashion), oxidizing pheophorbide *a* to colorless products, and therefore is the responsible for the loss of green color (*5*). It has been reported that this is the key regulatory step in the chlorophyll catabolism pathway (*4*). However, other oxidative enzymes such as lipoxygenase, chlorophyll oxidase, and peroxidase (*6*, *7*) seem to be also involved in the intermediate stages of the degreening process of chlorophylls (*8*).

During ripening and senescence, and coinciding with degeneration of chloroplasts into chromoplasts or gerontoplasts, a sharp decrease in the chlorophyll content is observed, but in most cases without accumulation of an appreciable amount of a particular catabolite, suggesting that there is a chain of degradative reactions in action. For this reason stay-green mutants of fruits (such as tomato and pepper) and vegetables such *Festuca pratensis* have shown to be very useful for investigating the biochemical basis of the degreening process (*9*). In the case of *Capsicum annuum* fruits, it has been demonstrated that chlorophylls are degraded during ripening in the same way as in the chloroplast of senescent leaves by the tamdem chlorophyllase, magnesium dechelatase and PaO (*10*). These authors found a high correlation between an increase in the PaO activity and disappearance of chlorophyll throughout fruit ripening. These results are also in accordance with the isolation of a primary fluorescent chlorophyll catabolite (Ca-FCC-2) produced in increasing amounts during fruit ripening (*11*). Chlorophyllase is present as a constitutive enzyme at all ripening stages, as part of the chloroplast−chromoplast membrane system, with higher values at green stages (*10*, *12*). In

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10.1021/jf060213t CCC: $33.50 
Published on Web 05/02/2006

the case of *C. annuum* fruits the chlorophyll retainer mutation (stay-green) is controlled by a single recessive gene, which causes inhibition of chlorophyll degradation during ripening (*13*). Molecular mapping of the chlorophyll retainer (CL) gene has been carried out recently in stay-green mutants of *C. annuum* (*14*). Surprisingly, whereas locus CL mapped on chromosome 1 of pepper (chromosome 8 in tomato), the corresponding loci for the enzymes involved in the early stages of chlorophyll catabolism mapped on different chromosomes, indicating that CL may correspond to a yet unknown gene from the chlorophyll catabolism pathway.

The aim of the present work is to investigate the chlorophyll catabolism pathway in five cultivars of *C. annuum* fruits, by the analysis of phytylated, dephytylated, and oxidized chlorophyll derivatives over the whole ripening period and differentiate both genotypes.

## MATERIALS AND METHODS

**Plant Material.** Fruits of the pepper (*C. annuum* L.) of selected lines (Mr 1, Mn 3, Rr 1, Rn 1, Rn 2, Lr 2, Lr 7, Dn 3, Dr 6, and Negral) were used for the present study. Plants were grown in open fields at the Centro de Investigación y Desarrollo Alimentario (CIDA, La Alberca, Murcia, Spain) except Negral variety plants, which were grown at the Escuela Técnica Superior de Ingenieros Agrónomos (Universidad de Castilla-La Mancha, Albacete, Spain). Both areas are in southeastern Spain and have similar climates. In general, the plantation density was 50000 plants/ha and under drip irrigation. Five to 10 fruits at different ripening stages were harvested every 15 days during the growing period of 75 days. Fruits were devoid of peduncles and seeds, cut into small pieces, and kept at −30 °C until analysis.

**Chlorophyll Extraction.** A 10 g sample of fruit was extracted with 30 mL of acetone, repeating the operation until no more color was extracted. The combined extracts (usually three or four times) were poured into a separating funnel and treated with 100 mL of diethyl ether. To this mixture was added 100 mL of sodium chloride (100 g/L); the ethereal phase was separated from the acetone one, washed three times with 100 mL of 50 g/L anhydrous $Na_2SO_4$, filtered through a bed of anhydrous $Na_2SO_4$, and evaporated to dryness under vacuum in a rotary evaporator (Büchi, Flawil, Switzerland) at 30 °C. The residue was dissolved in 5 mL of acetone and kept in a freezer at −20 °C.

**High-Performance Liquid Chromatography (HPLC).** This was carried out using an HP1100 Hewlett-Packard liquid chromatograph fitted with an HP 1100 automatic injector and a diode array detector. Data were collected and processed with an LC HP ChemStation (rev. A.05.04). A stainless steel column (25 × 0.46 cm), packed with 5 $\mu$m of C18 Spherisorb ODS-2 (Teknokroma, Barcelona, Spain), was used. The column was protected with a precolumn (1 × 0.4 cm i.d.) packed with the same material. The solution of pigments in acetone was centrifuged at 13000*g* (MSE Model Micro Centaur) prior to injection into the chromatograph (20 $\mu$L).

**HPLC Separation and Quantification of Chlorophylls.** Separation and quantification of the chlorophyll pigments was carried out following a method previously developed by the authors (*15*). Separation was performed using an elution gradient (flow rate = 2 mL $min^{-1}$) with the mobile phases (A) water/ion pair reagent/methanol (1:1:8 v/v/v) and (B) acetone/methanol (1:1 v/v). The ion pair reagent was 0.05 mol/L tetrabutylammonium acetate (Fluka, Chemie AG, Buchs, Switzerland) and 1 mol/L ammonium acetate (Fluka) in water. Detection was performed simultaneously at 666 nm for series *a* and 650 nm for series *b*. Response factors were calculated for each individual pigment by performing calibration plots (peak area ratio versus concentration ratio) in the presence of a known amount of the pure standard solutions. Chlorophylls (chl) *a* and *b* were purchased from Sigma. Chlorophyllide was formed by enzymatic de-esterification of chl. The reaction mixture contained 100 mmol/L Tris-HCl (pH 8.5) containing 0.24% (w/v) Triton X-100, chl *a* dissolved in acetone, and crude enzymatic extract from *Ailanthus altissima* (Mill.) leaves in a 5:1:5 (v/v/v) ratio (*16*). The C-13 epimer of Chl (*a* or *b*) was prepared by treatment with chloroform (*17*). $13^2$-OH-Chl (*a* or *b*) was obtained by selenium dioxide oxidation of Chl at reflux heating for 4 h in pyridine solution under argon (*18*). All Mg-free derivatives were obtained from the corresponding Chl parent dissolved in diethyl ether by acidification with 2−3 drops of 5 mol/L HCl (*19*). All standards were purified by normal phase (NP) and reversed phase (RP) thin-layer chromatography (TLC) (*15*, *20*).

**Statistical Analysis.** All experiments were carried out in triplicate. The coefficient of variation (CV) was calculated using Statistica for Windows (version 5.1, StatSoft, Inc., Tulsa, OK) and always was <10%.

**Table 1.** Description and Characterization of the Selected Lines of *C. annuum* (L.) Fruits

| variety | line | fruit shape | fruit position | phenotype (45 days) | phenotype (75 days) |
|---|---|---|---|---|---|
| M | Mn 3 | round | hanging | brown | red |
| | Mr 1 | elongated | erect | red | red |
| R | Rn 1 | round | hanging | brown | red |
| | Rn 2 | round | hanging | brown | red |
| | Rr 1 | round | | red | red |
| D | Dn 3 | elongated | hanging | brown | red |
| | Dr 6 | elongated | hanging | red | red |
| Lr | Lr 2 | elongated | hanging | red | red |
| | Lr 7 | elongated | hanging | red | red |
| Negral | | round | hanging | brown | brown |

## RESULTS AND DISCUSSION

**Evolution of the Total Chlorophyll Fraction.** The present study monitors wild-type lines of pepper that are red when ripe, as a consequence of the normal development of carotenogenesis, and mutant lines that are brown at normal harversting time (45 days). Within this latter group, the variety Negral maintains such coloring even in over-ripe fruit, whereas the other mutant lines become red at 75 days on the plant. The appearance of these mutant lines is the result of a spontaneous mutation, and—because of the commercial and scientific interest—they have been selected agronomically to stabilize the brown phenotype, distinguishing it from the lines that acquire the typical red color of ripe pepper. **Table 1** describes the samples analyzed in the present study, specifying the color of the fruits at the optimum moment of ripening for their harvesting (45 days), and the color acquired during over-ripening (75 days), which are the phenotype characteristics motivating the selection of the samples. Except for the variety Lr (lines 2 and 7) and the variety Negral, at least two lines of each variety have been analyzed: a mutant with brown phenotype and another acting as control within each variety in the case of intravarietal variations.

The analysis of changes in the total chlorophyll fraction (**Figure 1**) during ripening reveals great differences depending on phenotype. In the wild-type varieties, independently of the absolute level of chlorophylls in the fruits, the catabolic process of these compounds has finished before 45 days of ripening, and the concatenation of catabolic reactions prevents the detection of colored intermediaries during the process. In contrast, in the fruits of the mutant lines, chlorophyll degradation is much slower, these compounds being found in four lines even in the 75-day controls. The permanence in these lines of high levels of chlorophylls in parallel with the carotenogenic process explains their brown color at harvesting.

Chlorophyll degradation is always higher in the wild-type lines, and the differences become greater with ripening. The different behavior of the fruits of the variety Negral is particularly noteworthy: chlorophyll catabolism is almost

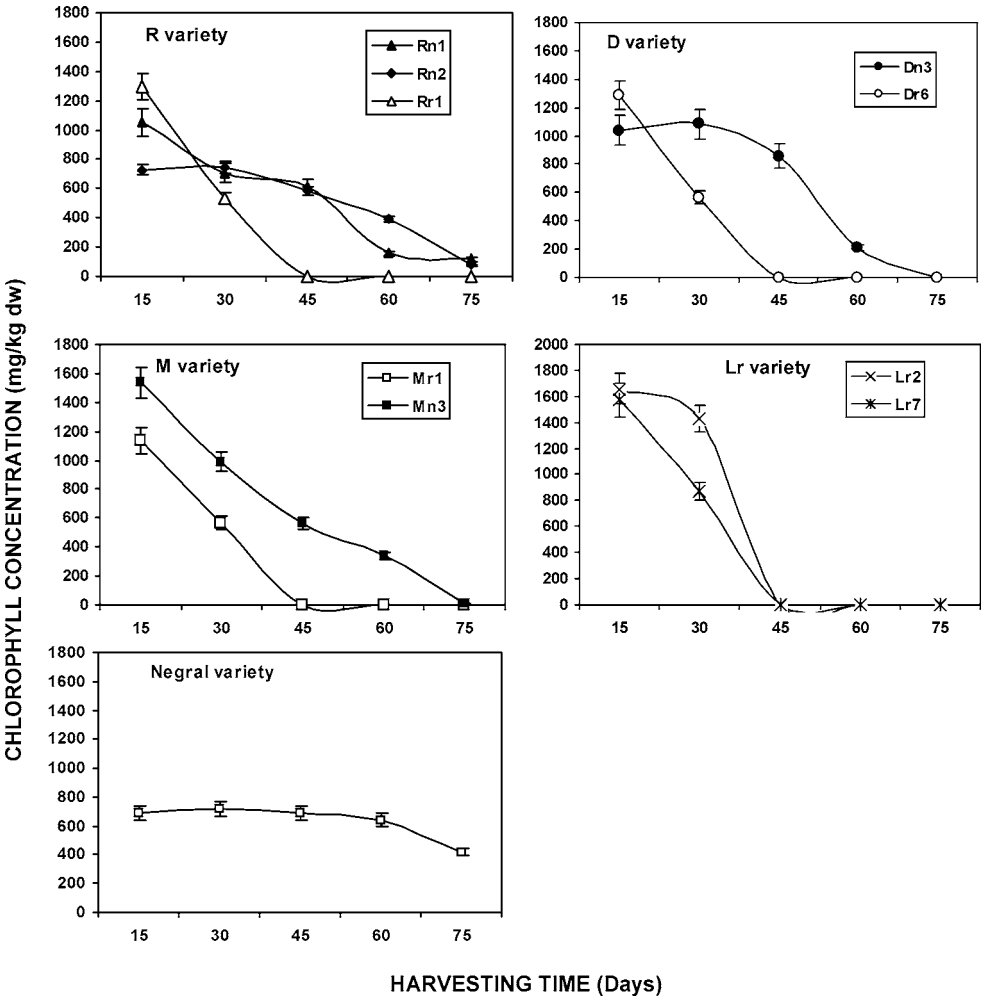


**Figure 1.** Changes in the total chlorophyll content (milligrams per kilogram of dry weight) of pepper fruits (*C. annuum* L.) during ripening on the plant of different lines from two genotypes: stay-green (solid symbols) and wild type (open symbols).

completely inhibited until 60 days of ripening, when it begins, with some 40% being lost by 75 days on the plant.

The term stay-green refers to an organism having delayed senescence (visually defined by the loss of chlorophylls) in comparison with that of a standard genotype (*9*). The five lines of brown pepper clearly show the effects of the mutation(s) responsible for the stay-green character.

**Relationship between Pigments and Structure/Function.** The chl *a*/*b* ratio is an indirect measurement of the distribution of centers of reaction/antenna complexes in the thylakoid and, thus, the degree of thylakoid packing (*21*). Studies at molecular level confirm the assumption that the antenna complexes are relatively rich in chl *b*, whereas the centers of reaction are rich in chl *a* (*22*). The chl *a*/*b* ratio in fruits has normally been reported to be around 2.5−4.0 units. The values obtained (**Figure 2**) for all of the lines analyzed (mutants and wild-type) are within the limits published for fruits, indicating that chlorophyll retention does not involve at the thylakoid structural level any alteration with respect to the standard. However, in the stay-green lines of the pepper, in late states of ripening (from 45 to 60 days), the ratio between the two chlorophylls shows a great increase. The exceptions are the fruits of the line Dn 3, which do not exhibit chlorophyll catabolites at the end of the study period and the variety Negral, which at the over-ripe stage (75 days) is comparable to the other stay-green lines at 30 days in terms of chlorophyll retention. This is in accord with recent theories (*23*) claiming that at an early point in the chlorophyll catabolic sequence, chl *b* prior to its degradation is transformed into chl *a*, probably by the enzyme chl *b* reductase. The increase in the ratio implies that the block in the chlorophyll catabolic pathway is located in reactions after the transformation of chl *b* into chl *a*. Thus, whereas chl *b* is degraded to chl *a* normally, the degradation of the latter is slowed, thereby altering the balance between them. In support of this hypothesis are the results obtained for the variety Negral, in which, due to the fact that the chlorophyll catabolism does not start (and then very slowly) until 60 days of ripening, the chl *a*/*b* ratio is not altered during the study period.

**Dephytylated Chlorophyll Catabolites.** The study of the chlorophyll composition shows the absence of chlorophyllides and pheophorbides in the wild-type lines, demonstrating that the rapid concatenation of metabolic reactions involved in the chlorophyll catabolic process prevents the accumulation of such catabolites. In contrast, in the stay-green lines, chlorophyllide *a* is found in the first states of ripening, later pheophorbide *a*, with pheophorbide *b* also detected in over-ripe fruits (**Table 2**). Assuming the PaO pathway in pepper fruits (*10*), in which chlorophyllase is active (*12*), the sequential accumulation of chlorophyllide—and later transformation to pheophorbide *a*—in the stay-green lines is indicative of a lesion at the level of PaO activity, per se, or in some modulator of the activity. The accumulation of chlorophyllide and pheophorbide implies that in these lines the enzymes chlorophyllase and magnesium dechelatase are functionally active.

The accumulation of dephytylated chlorophyll derivatives has already been reported for other organisms in which the mutation

**Table 2.** Oxidized and Dephytilated Chlorophyll Catabolites ($\mu$M/kg dw) in *Capsicum annuum* (L.) Fruits during the Ripening of Stay-Green Lines

Oxidized Chlorophyll Catabolites

| | Negral variety | | Line Mn 3 | | Line Rn 1 | | Line Rn 2 | | Line Dn 3 | |
|---|---|---|---|---|---|---|---|---|---|---|
| days | chl *a*[a] | chl *b*[b] | chl *a* | chl *b* | chl *a* | chl *b* | chl *a* | chl *b* | chl *a* | chl *b* |
| 15 | 0.81 ± 0.06 | – | 14.78 ± 0.93 | – | 85.42 ± 6.53 | 2.94 ± 0.15 | 87.70 ± 4.36 | 2.51 ± 0.35 | 8.02 ± 0.75 | 12.65 ± 0.54 |
| 30 | 3.97 ± 0.25 | – | 10.70 ± 0.54 | – | 109.99 ± 8.21 | 8.76 ± 0.64 | 103.72 ± 7.53 | – | 12.42 ± 0.65 | 0.63 ± 0.03 |
| 45 | 12.25 ± 0.82 | 9.67 ± 0.06 | 49.01 ± 0.24 | – | 68.09 ± 2.36 | 41.16 ± 1.25 | 72.16 ± 6.24 | 42.61 ± 7.53 | 148.47 ± 7.53 | 47.50 ± 7.53 |
| 60 | 37.46 ± 2.65 | 22.34 ± 0.65 | – | – | – | 6.61 ± 0.45 | 23.16 ± 1.98 | 27.78 ± 1.68 | – | – |
| 75 | 65.89 ± 4.85 | 26.62 ± 1.65 | – | – | – | – | – | – | – | – |

Dephytylated Chlorophyll Catabolites

| | Negral variety | | | Line Mn 3 | | | Line Rn 1 | | | Line Rn 2 | | | Line Dn 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| days | cda[d] | pha[e] | pb[e] | cda | pha | pb | cda | pha | pb | cda | pha | pb | cda | pha | pb |
| 15 | – | – | – | 1.61 ± 0.05 | – | – | 2.07 ± 0.08 | – | – | – | – | – | – | – | – |
| 30 | – | – | – | 4.75 ± 0.03 | – | – | | – | – | 2.51 ± 7.53 | – | – | – | – | – |
| 45 | – | – | – | – | – | – | – | – | – | – | – | – | 38.58 ± 1.67 | 14.95 ± 0.56 | 5.93 ± 0.25 |
| 60 | – | – | – | – | 61.29 ± 0.57 | – | – | 47.71 ± 2.68 | – | – | 38.00 ± 0.15 | – | – | 77.86 ± 7.53 | 7.36 ± 7.56 |
| 75 | tr[f] | 18.46 ± 0.16 | tr | – | 22.60 ± 1.68 | tr | – | 183.38 ± 10.98 | 8.22 ± 0.07 | – | 122.24 ± 7.53 | 7.85 ± 7.54 | – | – | – |

[a] chl *a*, 13$^2$-OH-chlorophyll *a*. [b] chl *b*:13$^2$-OH-chlorophyll *b*. [c] cda, chlorophyllide *a*. [d] pha, pheophorbide *a*. [e] pb, pheophorbide *b*. [f] tr, traces.

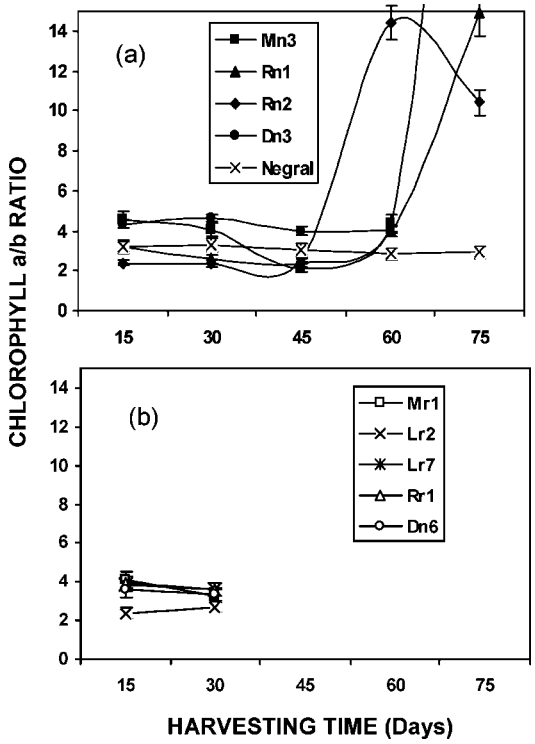


**Figure 2.** Changes in the chl *a*/chl *b* ratio of pepper fruits (*C. annuum* L.) during ripening on the plant of different lines from two genotypes: stay-green (**a**) and wild type (**b**). Symbols are as in **Figure 1**.

responsible for the stay-green character is related with PaO activity: leaves and fruits of the *gf* mutant in tomato, in which chlorophyllide *a* is accumulated (*24*); leaves of the *lls 1* mutant, in which pheophorbide *a* is accumulated (*4*); and senescent leaves of *Lolium perenne* (L.) (*25*), in which there is a sequential accumulation of chlorophyllide and pheophorbide. With regard to the pepper mutants, the most striking thing is that in three of the five lines, chl *a* disappears completely in the over-ripe fruit, becoming totally transformed into pheophorbide, the only chlorophyll derivative present at the last assay made. In studies of stay-green leaves of maize (*lls 1*) and *Arabidopsis* (*pao*), the mutation affects the PaO gene directly, and the pheophorbide *a* accumulated is only some 10% of the chlorophyll (*4*, *26*). This value is similar to that of dephytylated compounds accumulated in the stay-green mutant of *L. perenne* (L.) (*25*), and in all of them some 90% of the chlorophylls are in the form of chl *a* and *b*. A current postulation is the existence of feedback mechanisms, able to regulate the metabolism of chlorophylls in mutants unable to degrade chlorophyll beyond pheophorbide *a*, at the level either of chlorophyllase or of proteases involved in the degradation of chlorophyll-binding proteins. Such a pattern is shown by the stay-green lines of pepper in late states of ripening (60 days). However, if the study period is prolonged to 75 days, the chlorophyll is found to have been completely transformed into pheophorbide. Physiologically, the process of senescence in leaves has the general aim of recycling nutrients from parts of the plant that are no longer photosynthetically active while the fruits ripen to produce seeds. Although the processes of senescence and ripening are highly regulated in the plant and involve the controlled activation or inhibition of genes, the timing of senescence and that of ripening are very different. In the pepper, monitoring the changes in chlorophyll catabolites during ripening has enabled the study of a late state of ripening (over-ripe). To date, no such study could be made in leaves, so that it is not possible to establish a similar situation.

The unusual accumulation of pheophorbide *b* in the final state of ripening is an interesting point for study. As has been remarked in the previous section, the theory is accepted that chl *b* is transformed into chl *a* prior to its degradation (*23*), although it is possible that this takes place at the level of chlorophyllide *b*. However, the ring opening of a series *b* derivative by the enzyme PaO is not contemplated, as not only the specificity of PaO for the series *a* derivatives is demonstrated but so is the competitive inhibition of PaO with pheophorbide *b* (*5*). Therefore, it is postulated that although pheophorbide *b* is not formed naturally in the fruit during chlorophyll catabolism in the wild-type lines, in the mutants—with chl *b* present in late stages of ripening—magnesium dechelatase could act on chlorophyllide *b*.

It is particularly striking that the stay-green lines of pepper that have arisen randomly and spontaneously correspond to a mutation that affects PaO activity, above all when this enzyme is considered to be the critical, and most-regulated (pre- and possibly post-transcriptionally), point in the chlorophyll degradation pathway. Other stay-green lines have similarly arisen (*Festuca pratensis* Huds. or *Zea mays* L.), also affecting PaO activity. Although seemingly contradictory, it could be hypoth-

esized that the plant has developed protective mechanisms that counteract the negative effects of a possible mutation in one of its most important enzymes, thereby making these organisms viable.

**Oxidized Chlorophyll Catabolites.** The detailed identification and quantification of all of the chlorophyll catabolites generated during ripening in the lines under study enabled detection of the accumulation of oxidized chlorophyll catabolites, $13^2$-OH-chl *a* and *b* (**Table 2**), in the stay-green lines, which were absent from the wild-type lines. The accumulation of $13^2$-OH-chlorophyll describes a curve for which the maximum is reached at around 30−45 days, depending on variety, and then falls, except in the variety Negral, which shows a gradual increase parallel to ripening. In view of the possibility that chlorophyll oxidation is due to the fact that the chlorophylls are maintained in full senescence—much longer than normal—and that nonspecific oxidative mechanisms could oxidize these molecules, it has to be said that this nonspecific oxidation has not been observed in other affected stay-greens (*25*) that keep chlorophylls during senescence. It also seems (*4*, *22*, *24*) that the retention of chlorophylls during senescence in stay-green organisms stabilizes the proteins of the thylakoid pigment−proteolipid complexes. In fact, some stay-greens (such as *ore 10*) are mutations in proteases against LHCP II and, as a consequence, retain chlorophylls (*27*). It is therefore plausible to assume that the maintenance of these structures would protect the chlorophylls (as in green tissues) from nonspecific oxidation.

The enzymatic formation of $13^2$-OH-chl *a* was initially imputed to chlorophyll oxidase (*6*), but current research is looking more toward the involvement of peroxidase in this oxidation (*7*, *28*); this enzyme has been located in the chloroplast (*29*). The involvement of the hydroxylated derivative of chlorophyll as intermediate in chlorophyll catabolism during ripening has been reported in numerous fruits: satsuma mandarins (*8*), bananas (*30*), and olives (*31*). However, the exact role of this oxidation (the originating enzyme and the level of the pathway at which it participates) is still under debate. The results found in the stay-green lines of pepper confirm the existence of an oxidative mechanism that takes part in chlorophyll degradation and situate this oxidation in reactions prior to that catalyzed by the enzyme PaO, as chlorophyll retention in the stay-green lines affects the accumulation of oxidized compounds.

## ACKNOWLEDGMENT

We are sincerely grateful to IMIDA for supply samples.

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**Received for review January 24, 2006. Revised manuscript received March 16, 2006. Accepted March 17, 2006. M.R. was supported by a Ministerio de Ciencia y Tecnología contract (Juan de la Cierva).**

JF060213T