Chloroplast to chromoplast transition in tomato fruit: spectral confocal microscopy analyses of carotenoids and chlorophylls in isolated plastids and time-lapse recording on intact live tissue

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• *Background and Aims* There are several studies suggesting that tomato (*Solanum lycopersicum*) chromoplasts arise from chloroplasts, but there is still no report showing the fluorescence of both chlorophylls and carotenoids in an intermediate plastid, and no video showing this transition phase.

• *Methods* Pigment fluorescence within individual plastids, isolated from tomato fruit using sucrose gradients, was observed at different ripening stages, and an *in situ* real-time recording of pigment fluorescence was performed on live tomato fruit slices.

• *Key results* At the mature green and red stages, homogenous fractions of chloroplasts and chromoplasts were obtained, respectively. At the breaker stage, spectral confocal microscopy showed that intermediate plastids contained both chlorophylls and carotenoids. Furthermore, an *in situ* real-time recording (*a*) showed that the chloroplast to chromoplast transition was synchronous for all plastids of a single cell; and (*b*) confirmed that all chromoplasts derived from pre-existing chloroplasts.

• *Conclusions* These results give details of the early steps of tomato chromoplast biogenesis from chloroplasts, with the formation of intermediate plastids containing both carotenoids and chlorophylls. They provide information at the sub-cellular level on the synchronism of plastid transition and pigment changes.

Key words: Chloroplast, chromoplast, confocal, pigment fluorescence, *Solanum lycopersicum*, tomato.

INTRODUCTION

During evolution, chromoplasts have emerged as plastid structures which accumulate pigments to facilitate flower pollination and seed dispersal of fleshy fruit. There is good evidence that chromoplasts derive from chloroplasts (Pyke, 2007), even if nobody has ever recorded this transition. Structural changes occurring during chloroplast to chromoplast transition have been described in fleshy fruit by electron microscopy primarily in tomato (Rosso, 1968; Harris and Spurr, 1969) and in bell pepper (Spurr and Harris, 1968). During the differentiation process controlled breakdown of chlorophyll and disruption of the thylakoid membrane occurred, concomitant with an increase in the aggregation of carotenoids. Different carotenoid-accumulating bodies have been described, including plastoglobules, crystalline and microfibrillar structures, and internal membranous structures (Marano *et al.*, 1993). Coloured images have been made under bright-field microscopy of plastids of fruit and flowers where chloroplasts appear as dark green pigmented bodies, while chromoplasts appear as dark red or orange bodies (Gunning, 2005). More recently, Pyke and co-workers (Pyke and Howells, 2002; Waters *et al.*, 2004; Forth and Pyke, 2006; Pyke, 2007) have studied chloroplast to chromoplast transition using green fluorescent protein (GFP) constructs targeted to the plastid by the RecA plastid transit sequence. This technique showed the highly irregular and variable morphology of plastids between different types of cells. It also allowed the morphology of stromules to be studied and the presence of bead-like structures along the stromules to be discovered. In Pyke and Howells (2002), the fluorescence of carotenoids might have been quenched by the use of an anti-fading compound, Vectashield® (http://forums.biotechniques.com/viewtopic.php?f=17&t=22802); therefore, chromoplasts were observed in ripe fruit tissues using the merging of GFP fluorescence and bright-field images, but the transition from chloroplasts was not observed. In later studies (Waters *et al.*, 2004; Forth and Pyke, 2006), the authors focused on chlorophyll and GFP fluorescence during the transition from chloroplasts to chromoplasts and then onto stromules. No observation with carotenoid fluorescence was performed. In the present work, a laser scanning confocal microscopy technique has been used to monitor the loss of chlorophylls and the accumulation of carotenoids simultaneously in individual plastids isolated at different stages of development. Time-lapse recording on tissue slices of tomato fruit was also performed to follow the transition *in situ* in a cellular set of plastids.

MATERIALS AND METHODS

Plant material

Tomato plants (*Solanum lycopersicum* 'MicroTom') were germinated and cultivated under greenhouse conditions and fruit was collected at the mature green, turning (2 d after breaker, Br + 2) and ripe (10 d after breaker, Br + 10) stages as shown in Egea *et al.* (2010). The breaker stage is characterized by the initiation of fruit colouration, with fruit changing from green to pale orange at the blossom end.

Plastid isolation and intactness assessment

Fruit were thoroughly washed with distilled water, the seeds and gel were eliminated and the pericarp was cut into small pieces (0·5–1·0 cm). Prior to homogenization, small fruit pieces were incubated in ice-cold extraction buffer (250 mM HEPES, 330 mM sorbitol, 0·5 mM EDTA, 5 mM β-mercaptoethanol, pH 7·6) for 30 min. Intact purified plastids were obtained by differential and density gradient centrifugation in discontinuous gradients of sucrose, as previously described by Barsan *et al.* (2010) with some modifications. Fruit for extraction of chloroplasts, immature chromoplasts and mature chromoplasts were chosen from mature green, breaker and ripe fruit, respectively, as described above. For isolating chloroplasts, a three-layer sucrose gradient was used, 0·9 M–1·15 M–1·45 M, and for mature chromoplasts the sucrose gradients were 0·5 M–0·9 M–1·35 M. To obtain intact purified immature chromoplasts, from breaker fruit, a more sensitive discontinuous sucrose density gradient was necessary (0·5 M–0·9 M–1·15 M–1·25 M–1·35 M–1·45 M). Intact chloroplasts, immature chromoplasts and mature chromoplasts banded in the 1·15 M–1·45 M, 0·9 M–1·15 M and 0·9 M–1·35 M sucrose interfaces, respectively. The plastid bands collected were washed twice with extraction buffer and finally resuspended in extraction buffer for further confocal microscopy observations.

For the intactness assessment, the isolated plastids were suspended in a buffer containing 25 mM Bicine, 25 mM HEPES, 2 mM $MgCl_2$, 2 mM dithiothreitol, 0·4 M sorbitol, pH 9 supplemented with an equal volume of carboxyfluorescein diacetate (CFDA) at a final concentration of 0·0025 % (w/v) and incubated for 5 min (Schulz *et al.*, 2004). CFDA fluoresces strongly when it is de-esterified to carboxyfluorescein in an intact plastid. Plastid suspensions were examined using an inverted microscope (Leica DMIRBE) equipped with an I3 cube filter (excitation filter 450–490 nm, dichroic mirror 510 nm and emission filter LP 515 nm). The number of total plastids per microlitre in the samples was determined using a haemacytometer (Neubauer Double, Zuzi), and the results were expressed as a percentage of intact plastids.

Tomato mesocarp preparation for in situ *time-lapse recording*

Very thin slices (around 300 μm) of tomato mesocarp were hand cut with a razor blade on the green part of inner + outer mesocarp layers, at the turning stage (Br + 2). Slices were mounted on a glass slide in water (as shown in Supplemenatary Data Fig. S1A, available online), containing 0·1 mM of the ethylene precursor 1-aminocyclopropane-1-car boxylic acid (ACC). This treatment enhanced the chance of observing colour changes within a time interval (several hours), during which the tissues neither moved on the slide nor dehydrated.

Confocal microscopy of isolated plastids

Confocal images of isolated plastids from the different fractions were acquired with a laser scanning confocal system (Leica LSCM-SP2, Nanterre, France) coupled with an upright microscope (Leica DM6000, Rueil-Malmaison, France). Samples of freshly isolated plastid fractions were placed between a glass slide and a coverslip. Fluorescence emission spectra were acquired using the 488 nm ray line of an argon laser for excitation and the emitted fluorescence was recorded from 505 to 745 nm with a bandwidth of 10 nm using the λ-scan module of the Leica software. The fluorescence intensity expressed with arbitrary units corresponds to the average intensity of fluorescence per pixel.

Confocal microscopy for time-lapse recording on mesocarp tissues

Time-lapse acquisitions were performed using a long working distance ×40 water immersion lens (NA: 0·8) dipping directly in the buffer. Slices of the mesocarp tissue were placed at the bottom of a Petri dish filled with buffer to avoid both displacement and dehydration during the time-lapse acquisition (shown in Supplementary Data Fig. S1B). A hole in the cover of the Petri dish allowed the objective lens to dip in the buffer. The whole apparatus (Petri dish and objective lens) was covered with Parafilm® to avoid dehydration. The 488 nm excitation ray line of an argon laser was used to image the distribution of carotenoids, the emitted fluorescence being collected between 500 and 600 nm. For chlorophylls, the 633 nm excitation ray line of a He:Ne laser was used and the emitted fluorescence between 650 and 700 nm was collected. Over 18·5 h, images were acquired every 15 min taking advantage of the time-lapse module of the Leica software. To reduce photodamage due to laser illumination, both laser intensities were minimized, and image averaging was not performed. To collect fluorescence emission, the pinhole was opened at Airy = 2 and the photomultiplier gain increased, which generated some electronic noise, especially for the carotenoid channel. Overall, the photobleaching was reduced during image acquisition. Measurements of fluorescence intensity were performed on the raw t-series with Image-Pro software. There was more variability in the carotenoid signal than in the chlorophyll signal. This may be due to a differential setting of the photomultiplier in the two different channels. As an increase in carotenoid signal was expected, to avoid

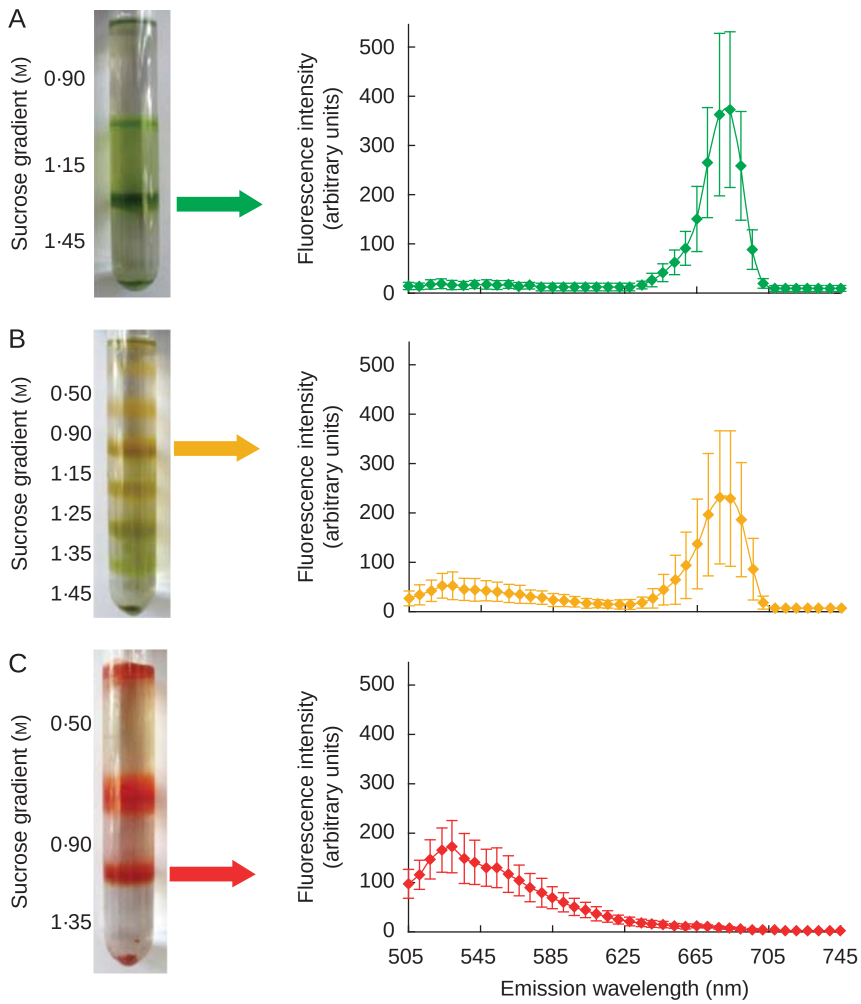

FIG. 1. Separation on a sucrose gradient of tomato plastids at three stages of fruit ripening and corresponding fluorescence emission spectra. Plastid fractions from tomatoes at the mature green (A), breaker + 2 d (B) and breaker + 10 d (C) stages have been analysed by a laser confocal scanner to generate fluorescence emission spectra of individual plastids. Fluorescence intensity of the fractions indicated by an arrow is given as arbitrary units ± s.d. ($n > 50$). The excitation wavelength was 488 nm. The peaks of fluorescence emission around 520 and 680 nm correspond to carotenoids and chlorophylls, respectively.

saturation at the end of the time-lapse recording, the photomultiplier was adjusted to a low level of amplification, corresponding to a low signal/noise ratio. This was not required for the chlorophyll channel, where a decrease was expected, and for which the signal was already high at the beginning of the time-lapse recording.

RESULTS AND DISCUSSION

Characterization of chloroplast to chromoplast transition in isolated plastids with a focus on the intermediate stages

Plastids were isolated at different development stages and separated on sucrose gradients. The degree of integrity of plastids in the fractions was analysed with a fluorescent dye, CFDA (Schulz *et al.*, 2004). The intactness of plastid fractions retained for further analysis (indicated by arrows in Fig. 1A–C), reached between 85 and 90 %, 80 and 85 %, and 65 and 70 %, respectively. The upper layers in the gradients contained broken plastids.

At the mature green stage, intact green plastids were localized in a single band at the 1·15–1·45 M sucrose interface (Fig. 1A). Fluorescence confocal analyses of the plastid pool in this band indicated the almost exclusive presence of chlorophylls, characteristic of chloroplasts (Fig. 1A). This was confirmed by a spectrophotometric analysis of pigment absorbances (Supplementary Data Fig. S2).

Plastids isolated from fruit at the breaker stage were separated into four layers in a more fragmented sucrose gradient, excluding broken plastids in the upper part of the gradient above 0·9 M sucrose (Fig. 1B). The presence of different fractions of plastids with different colours from green to yellow clearly indicates that the differentiation process is not occurring synchronously. It is well known that chloroplasts have a greater density and a smaller size than chromoplasts (Rosso, 1968; Iwatzuki *et al.*, 1984; Hadjeb *et al.*, 1988). A good illustration of the decrease in density during the transition from

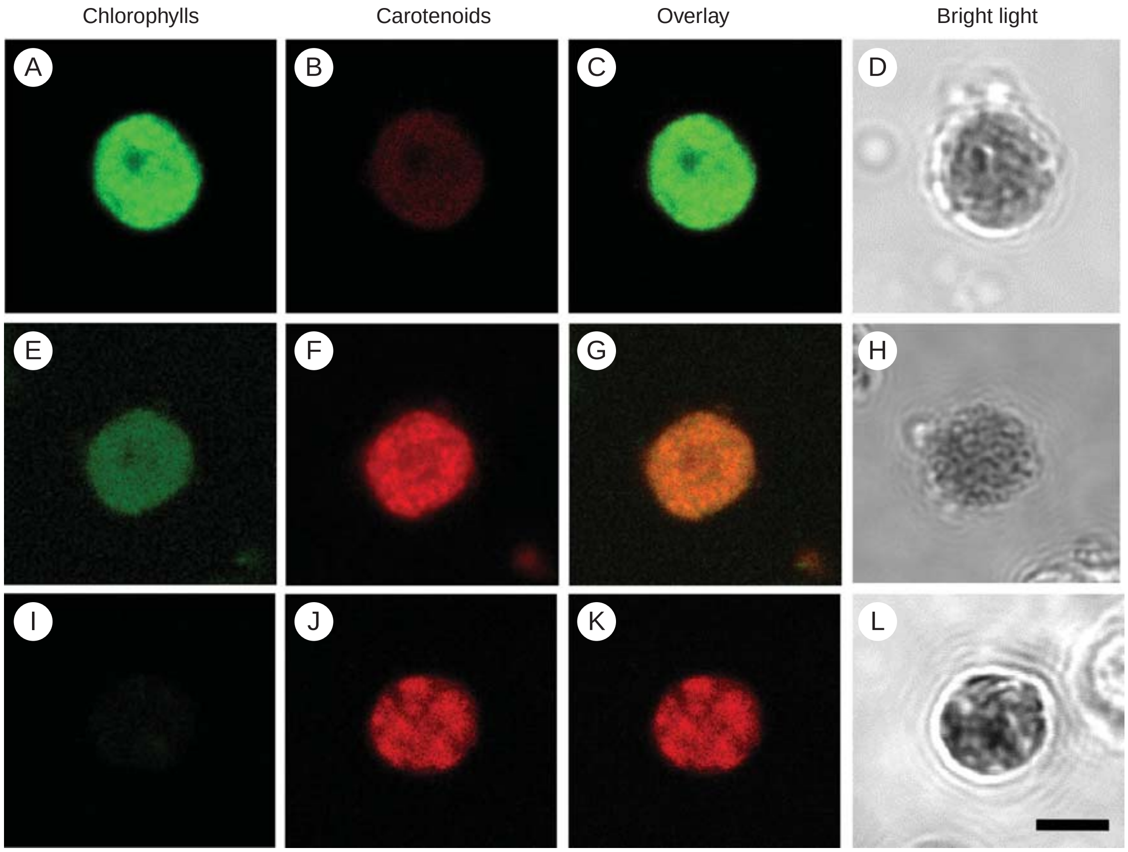

FIG. 2. Confocal microscopy of tomato plastids isolated at three stages of fruit ripening. (A–D) Chloroplasts isolated at the mature green stage. (E–H) Immature chromoplasts isolated at the turning stage (breaker + 2 d). (I–L) Chromoplasts isolated at the fully ripe stage (breaker + 10 d). (A), (E) and (I) correspond to chlorophyll fluorescence emitted from 650 to 750 nm; (B), (F) and (J) to carotenoid fluorescence emitted from 500 to 600 nm; and (C), (G) and (K) to the overlay of chlorophyll and carotenoid autofluorescence of the same plastid images. (D), (H) and (L) correspond to images of transmitted light. Chlorophyll fluorescence is shown in green and the carotenoid fluorescence is shown in red. Scale bar = 4 µm.

chloroplasts to chromoplasts is shown in Fig. 1B, where a range of intermediate plastid forms are visible. After confocal microscopy analysis of the fractions, only the 0·9–1·15 M fraction contained a majority of plastids in a transient stage between chloroplast and chromoplast. Confocal analyses (Fig. 1B) showed that this fraction contained reduced levels of chlorophyll and significant amounts of carotenoids. This result was confirmed upon extraction of pigments and spectrophotometric analyses (Supplementary Data Fig. S2) and is typical for chromoplasts at the early stages of differentiation (also called immature chromoplasts). The other fractions below 1·15 M sucrose were more heterogeneous and comprised both early developing chromoplasts and chloroplasts (data not shown). Each band of the gradient was analysed for its chlorophyll and carotenoid content by solvent extraction (Supplementary Data Fig. S2) and the band at the interface of 0·9–1·15 M sucrose harboured a ratio close to 1:1 of chlorophylls:carotenoids. To our knowledge, intermediate plastids, in transition from chloroplast to the chromoplast stage, as shown in Fig. 2E–H, have never been characterized in previous works dealing with the isolation of chromoplasts from fruit pericarp (Siddique *et al.*, 2006; Martí *et al.*, 2009). Plastids from fully ripe fruit were separated in a single band at the 0·9–1·35 M interface (Fig. 1C) and contained almost exclusively carotenoids, typical of chromoplasts.

Confocal observations of individual plastids provided more insight into the pigment transition at the plastid level (Fig. 2). The chloroplasts obtained from green tomatoes (Fig. 1A) were observed by confocal microscopy imaging, and a representative plastid is shown in Fig. 2A–D. It emitted fluorescence mostly in the chlorophyll emission range (Fig. 2A), and weakly in the carotenoid range (Fig. 2B), so that the carotenoid fluorescence emission is masked by chlorophyll fluorescence in the overlay picture (Fig. 2C). A typical plastid of the breaker stage (Fig. 2E–H), taken from the orange band of Fig. 1B, exhibited significant fluorescence of both chlorophylls (Fig. 2E) and carotenoids (Fig. 2F), giving an orange colour in the overlay picture (Fig. 2G). This plastid represented an intermediate differentiation stage between chloroplast and chromoplast. The suspension of chromoplasts isolated from red fruit contained only fully differentiated chromoplasts (Fig. 2I–L), that emitted fluorescence only in the carotenoid emission range (Fig. 2J), and not at the specific wavelength of chlorophylls (Fig. 2I). All isolated plastids were spherical; this may be due to the fact they were removed from the cell where it has been observed that stromules (Waters *et al.*, 2004) and

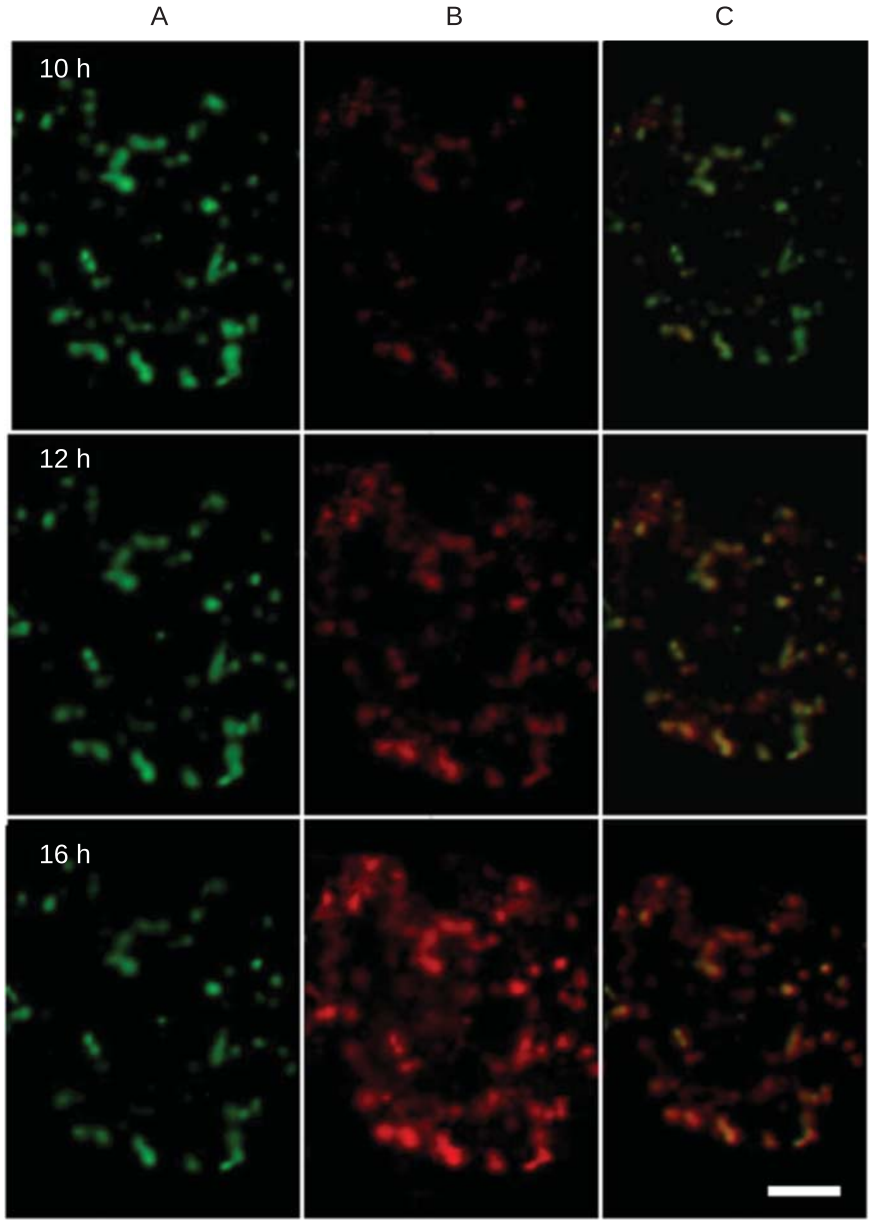

FIG. 3. Time-lapse evolution of chlorophyll and carotenoid fluorescence by confocal microscopy of plastids from tomato fruit mesocarp cells at the early stages of the transition from chloroplast to chromoplast. (A) Chlorophyll fluorescence, (B) carotenoid fluorescence and (C) overlay of both images at 10, 12 and 16 h. The emitted fluorescence was collected between 650 and 750 nm for chlorophyll and between 500 and 600 nm for carotenoid. The timing corresponds to the arrow shown in Fig. 4. A video showing the kinetics of these variations is available in supporting information (Supplementary Data, Video S1). Chlorophyll fluorescence is shown in green, and carotenoid fluorescence is shown in red. Scale bar = 50 μm.

microfilaments and microtubules (Kwok and Hanson, 2003) control plastid morphology.

In situ real-time recording of the chloroplast to chromoplast transition

To get a complete sequence of the changes occurring at the beginning of the pigment switch, a real-time recording of mesocarp tissues was performed (Supplementary Data Video S1). Pictures extracted from the video (Fig. 3) show that the number of plastids per cell of the 'MicroTom' cultivar is around 70, which is about the number of plastids per cell observed in the inner mesocarp of 'Ailsa Craig' (Waters *et al.*, 2004). However, there also appeared to be differences between the two cultivars. During the transition, the fluorescence of carotenoids increased steadily within the plastids within 6 h (Fig. 3B). Interestingly, in these conditions, where observations could be made at the cell level, it appeared that the transition was rather synchronous, as shown by the presence of a limited number of plastids having different colours upon merging of the spectral views of chlorophyll and carotenoids (Fig. 3C). This is in contrast to the large number of intermediate plastid forms observed at the breaker stage in the isolated plastid population arising from whole tissues

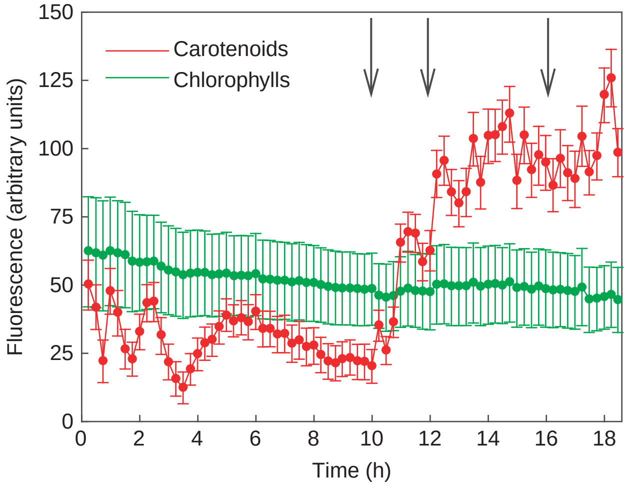

FIG. 4. *In situ* kinetics of chlorophyll and carotenoid fluorescence emission by confocal microscopy of plastids of tomato fruit mesocarp cells at the early stages of the transition from chloroplast to chromoplast. Intensity measurements were made on a selection of 150 individual plastids from four different cells. Error bars represent the s.d. The arrows show the timing of the pictures in Fig. 3.

(Fig. 1B). Although in the present experiment the ripening process was accelerated by the addition of ACC, it can be assumed that the synchrony of transition from chloroplasts to chromoplasts was higher within a cell than between cells of the fruit tissue.

The ratio of chlorophyll to carotenoid fluorescence was calculated for a total of 70 plastids present in a single cell at 10 and 16 h, by considering that a ratio >1 corresponded to chloroplasts, and a ratio <1 to chromoplasts. Over the 6 h of image recording, 84·3 % of the fluorescing plastids turned from chloroplasts to chromoplasts, 8·6 % stayed as chloroplasts and 7·1 % were already chromoplasts at the beginning of the observation period. Therefore, >80 % of the plastids have undergone a transition within 6 h, which is indicative of strong intracellular synchrony. Interestingly, there was no appearance of new plastids, thus confirming that all chromoplasts derived from pre-existing chloroplasts, as suggested by Pyke and Howells (2002) and Waters *et al.* (2004).

The recording of fluorescence emission of chlorophyll and carotenoids showed that very little change occurred during the first 10 h (Fig. 4). At 10 h, the carotenoid fluorescence suddenly increased, while chlorophyll fluorescence decreased more slowly. The increase in carotenoid fluorescence was 2·5-fold in the first 20 h interval, while the decrease in chlorophyll fluorescence was at most 0·25-fold during the same period. After 14 h, the fluorescence of carotenoids reached a maximum and stayed constant until the end of the observation period. This is in contrast to whole fruit that continue to accumulate carotenoids over a week after the breaker stage (Fraser *et al.*, 1994). The excision and survival of fruit tissues may be responsible for the early cessation of carotenoid accumulation beyond 14 h (Fig. 4); however, it is also possible that the cell has reached its maximum capacity of carotenoid accumulation. Indeed, it seems that the continuous accumulation of carotenoids in fruit tissues is the sum of carotenoid content of thousands of cells, which gradually turn red. The possibility that chlorophyll degradation products could exhibit fluorescence and therefore unmask chlorophyll breakdown should be excluded. It has been demonstrated that chlorophyll breakdown products do not accumulate in higher plants (Matile *et al.*, 1999) and they are rapidly metabolized into non-fluorescent compounds (Oberhuber *et al.*, 2003). Our data on pigment changes are in agreement with previous observations showing that lycopene accumulated at a rate that was between three and four times higher than the corresponding chlorophyll decline (Trudel and Ozbun, 1970; Wu and Kubota, 2008). The very fast increase in carotenoid accumulation can be related to a very strong increase in the expression of phytoene synthase and phytoene desaturase genes between the mature green and breaker stages (Giuliano *et al.*, 1993; Ronen *et al.*, 1999).

The biogenesis of plastids, more particularly the conversion of chloroplasts into chromoplasts, has become a major field of interest among plastid physiologists (Pyke, 2007; Kahlau and Bock, 2008; Egea *et al.*, 2010). In the present work, laser scanning confocal microscopy has been used to study, at a subcellular resolution, the biogenesis of chromoplasts resulting from the conversion of chloroplasts in tomato fruit. The changes in carotenoids and chlorophylls have been monitored simultaneously on both isolated plastids and intact live mesocarp tissues. By recording the kinetics of short time changes, this method has allowed a fine description of the early steps of the transition to be described. The transition started in the nascent chromoplast by a sharp accumulation of carotenoids while the chlorophyll level was still high. The transition was more synchronous within plastids of a single cell than between cells of the fruit tissue. The real-time monitoring of the transition presented in a video revealed that all the chromoplasts derived from pre-existing chloroplasts in mesocarp tissues.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Video S1: pigment changes that occur during the early stages of the transition from chloroplast to chromoplast in mesocarp tomato cells, as viewed under a laser scanning confocal system (Leica LSCM-SP2) coupled with an upright microscope (Leica DM6000); the video is available in both .wmv and .avi formats. Figure S1: experimental device used to make the video. Figure S2: ratio of chlorophylls to carotenoids in isolated plastids.

ACKNOWLEDGEMENTS

We thank Katerynne Garcia and Sharon Poule (students at the University of Toulouse) for their help in preparing the tomato slices and recording experiments. We thank Dr Ross Atkinson (Plant & Food Research, Auckland, New Zealand) for fruitful suggestions. We are grateful to the referees and editors for the time spent improving our work. This work was supported by the National Natural Science Foundation of China (grant number 31071798) and the Midi-Pyrénées Regional Council (grant number CR07003760).

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