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This is author's accepted manuscript. The final published article can be cited as Konagaya, K., Al Riza, D. F., Ogawa, Y., Kohno, Y., Kuramoto, M., Takahashi, N., Tetsuhito Suzuki, Kondo, N., Autofluorescence changes of tomato surface tissues during overripening. Photochemical & Photobiological Sciences, 19(7), 879-884, 2020.

COMMUNICATION

Autofluorescence changes of tomato surface tissues during overripening

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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We investigated the autofluorescence of tomato surface tissues during overripening at 25 °C for 13 days. Microscopic images and fluorescence spectra of tissues, including the epidermis and cuticle, were examined (excitation at 360 nm), revealing that the autofluorescence changes were related to the epidermis, particularly the fluorophores in the cuticle.

Introduction

Tomato autofluorescence can change even after the colour change is saturated; thus, it can be used as an index of overripening.[1,2] In the ultraviolet-visible ranges, intense fluorescence was observed in the excitation range of 360−370 nm (i.e. in the long wave ultraviolet A (UVA) region of 310−400 nm) when conducting non-destructive measurements,[2,3] which enable the inspection of all products.

To employ this non-destructive technique in practical applications, tomato tissues, which present changes in autofluorescence during overripening, should be investigated. In general, tissue functions (i.e. barrier,[4] mechanical strengthening,[5] and moisture storage[6]) are supported by the chemical compositions and some of them are autofluorescent. Therefore, the investigation of the autofluorescence properties of the tissues during overripening can lead to tissue function assessment.

In this study, we investigated tomato tissues that underwent autofluorescence (excitation at 360 nm) during overripening. The distribution of fluorophore was examined using fluorescence microscopic images at a longitudinal section (excitation at 365 nm). Moreover, fluorescence emission spectra in four tissue samples were measured; these samples had varying thicknesses (32 µm–3 mm), achieved by removing the inner cell layers. The origin of the autofluorescence changes in the tissue surface was discussed in terms of the location of the fluorophores.

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Results

Tomato (*Solanum lycopersicum* L.) plants (cultivar Momotaro) were grown in a semi-open greenhouse in Ehime, Japan. After harvest, the tomatoes were stored in a dark incubator for 13 days at 25 ± 1 °C. Before proceeding to the tissue investigation, we confirmed that the autofluorescence shown in the intact tomato images on day 1 was different from that on day 13 (Fig. S1). At day 1, the fluorescence image was dark blue; however, it changed to whitish blue at day 13, as reported previously.[2] Subsequently, we destructively investigated the locations where the autofluorescence changes occurred.

To investigate the distribution change in the fluorophore at the longitudinal sections at both ends, fluorescence microscopic images were obtained. Fig. 1 depicts the two exemplar autofluorescence images acquired on days 1 and 13 (not shown for day 7). On both days, fluorophores were localized within 50 µm from the surface. This structural scale is equivalent to one or three epidermal cell layers. Anatomically, the outermost cell layer consists of epidermal cells (20–30 µm in the depth direction), the subsequent one or two cell layers are sub-epidermal cells (20–100 µm in the depth direction), and the several inner cell layers are referred to as hypodermal cells or collenchyma cells (40–340 µm in the depth direction), which integrally form the peel tissue called the exocarp (approximately 100–500 µm in the depth direction) together with the epidermis. The fluorescence width (50 µm in the depth direction) was more similar to the thickness of the epidermis (approximately 70 µm) than that of the peel tissue (around 260 µm), as presented in Table S1. The outermost one to three cell layers of the epidermis emitted a dark-blue or whitish-blue autofluorescence during the storage.

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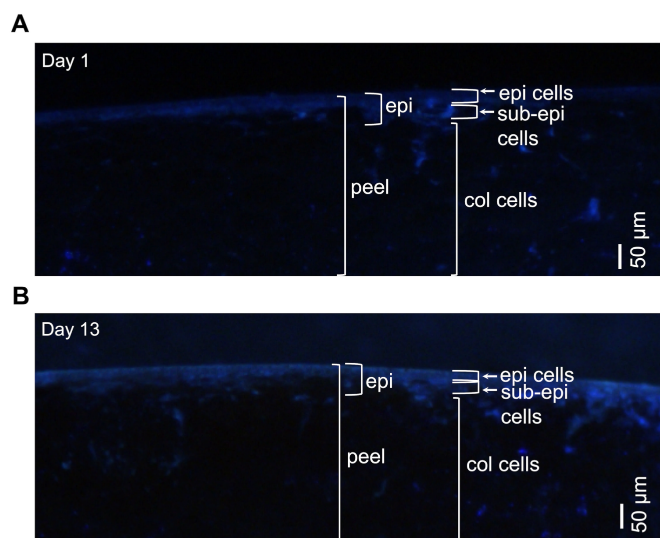

Fig. 1 Microscopic image of tomato emitting bluish fluorescence under excitation at 365 nm. (A) tomato at harvest and (B) after storage at 25 °C on day 13. The black-blue interface observed in the upper fourth of each image indicates the surface of the tomato, and its flesh is beneath this interface. The boundary of each part is approximated based on the scale (see Table S1). The image depends on the RGB sensitivity.

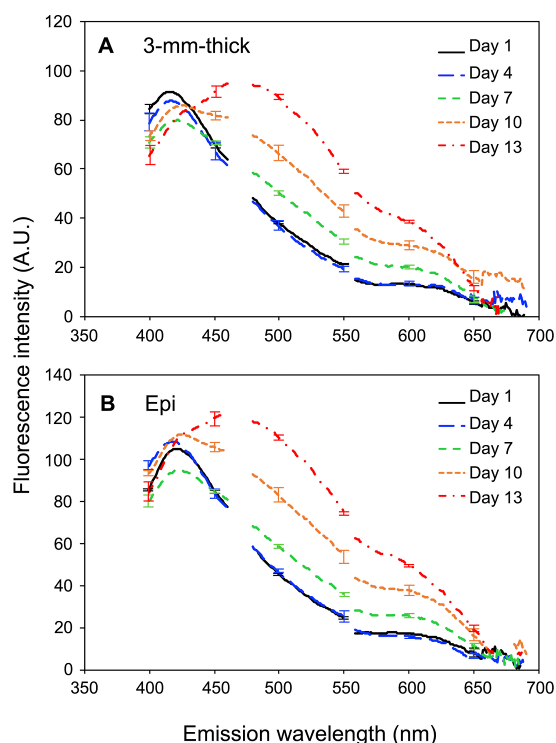

Fig. 2 Fluorescence emission spectra of (A) 3-mm-thick tomato sample and (B) epidermis (epi) during storage at 25 °C until day 13. The excitation wavelength is 360 nm. The error represents the standard error of three fruits.

To investigate the tissue that emitted autofluorescence during overripening, four types of tissues (Table S1, Fig. S2) were separated and their fluorescence spectra were examined. To confirm the validity of each tissue, the thickness was measured at three points of approximately 30 samples of 6 mm diameter in each tissue using a micrometre (Mitutoyo Corp., Japan), as shown in Table S1. The spectra were measured using a spectrofluorometer. Fig. 2 depicts the fluorescence emission spectra of a 3-mm-thick tomato sample and epidermis. The mean thickness of the sample and epidermis was 3 mm and 70 µm, respectively. In the surface normal direction from 3 mm down to 70 µm, the fluorescence spectral intensity and shape were almost consistent. The spectra exhibited a peak at 420 nm, and it broadened with another peak at 470 nm. The broadening corresponded to the change in fluorescence emission from blue (400–500 nm in half-width) to whitish-blue (400–550 nm in half-width) as observed in the images (Fig. 1). The spectral data further assured that the autofluorescence on the surface was mainly caused by the epidermis.

The fluorescence spectrum of the cuticle was further examined to attribute the change in the epidermal fluorescence to its constituents: the cuticle or the cells (i.e. epidermal cell and sub-epidermal cell). Fig. 3A shows the fluorescence spectra of the isolated cuticle. The spectra exhibited two peaks at 420 nm and 540 nm. In particular, the peak at 540 nm increased. The increase of fluorescence emission in the range of 430–650 nm was the same characteristic as that observed in the epidermis (Fig. 2B). However, the fluorescence spectra of the epidermis and cuticle were considerably different. To clarify this, the fluorescence spectrum difference was calculated (Fig. 3B). The fluorescence intensities increased when the cuticle was isolated by removing the cellular constituents from the epidermis. Thus, the spectrum obtained by subtracting the cuticle from the epidermis was negative. The minimum value was found at approximately 540 nm. In addition, a baseline-shaped reduction, which was stronger at shorter wavelengths, was found.

The higher fluorescence intensities of the cuticle, compared with those of the epidermis, could be attributed to the existence of the epidermal and sub-epidermal cells. We inferred this phenomenon based on the structures of the cuticle and epidermis (Fig. S3). The cuticle is a tissue where cutin and wax are deposited in the cell walls of the outermost one to three cell layers. Several previous studies have reported on autofluorescent phenolics in the cuticle by using DPBA fluorescent probes,[7] TLC,[8] and GC-MS,[9–11] although fluorescence was only investigated by Laguna *et al.*[8] Based on the cuticular structure, the excitation light penetrating into the epidermis should be attenuated by the cells before reaching the fluorophores (Fig. S3). In addition, emission light from the cuticle might also be attenuated by the cells, which are surrounded by the cuticle.

The higher attenuation factor of the epidermis compared with that of the dried cuticle (empty cuticle) may consist of scattering and absorption. Fluorescence reduction (Fig. 3B) showed a decrease at 540 nm and a baseline-like reduction effect at the sorter wavelengths. This suggests the following: pigment(s) have a maximum absorption at 540 nm and an increased scattering occurs at shorter wavelengths. The

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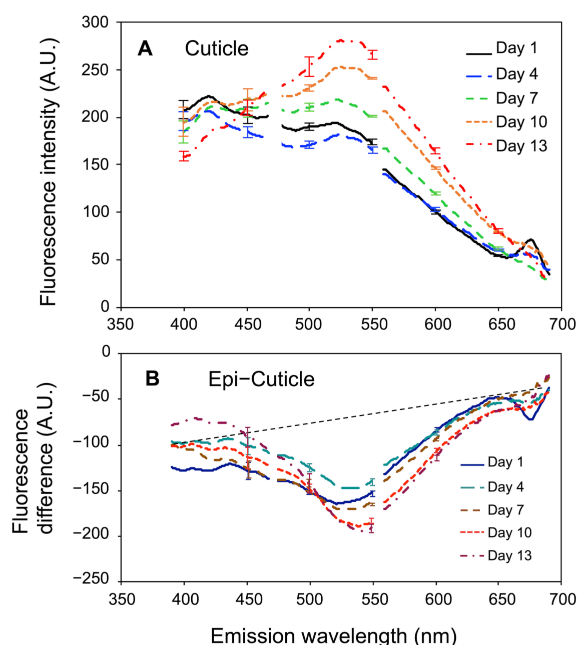

Fig. 3 (A) Fluorescence emission spectra of the cuticle. (B) Fluorescence emission spectra difference of the epidermis (epi) and the cuticle. The tomatoes are stored at 25 °C until day 13. The excitation wavelength is 360 nm. The dashed black line indicates a baseline-shaped reduction. The error represents the standard error for three fruits.

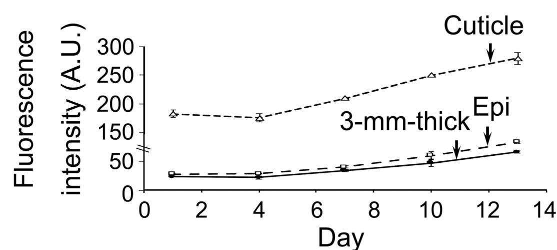

Fig. 4 Change in fluorescence intensity at 540 nm for the 3-mm-thick tomato sample, epidermis (epi), and cuticle. Tomatoes are stored at 25 °C until day 13. The excitation is at 360 nm. The error bar represents the standard error for three fruits.

existence of green absorbing pigments in the cells was also observed by the colour difference between the two tissues (Fig. S4). Initially, the epidermis was red (hue = 28.9 ± 0.9°); however, after the removal of the cell layers, the cuticle became yellow[7,9,12] (hue = 70.7 ± 1.6°), when measured using a colorimeter. This indicates that both tissues contain the blue absorbing pigments (e.g. yellow flavonoids in the cuticle[7,9,12]), but only cells contain green absorbing pigment(s) such as lycopene that have a peak maximum at approximately 550 nm[13] in the intact tissue. As the cell layers in the epidermis only demonstrated the fluorescence reduction contribution, the fluorescence emission was assigned to fluorophores in the cuticle.

To further investigate if the increase in the fluorescence emission centred at 540 nm in the 3-mm-thick sample can be attributed to the cuticle, the change in the fluorescence intensity at 540 nm was plotted for each tissue (Fig. 4). It was observed that, in the cuticle alone, fluorescence intensity at 540 nm increased similarly to those of the epidermis and the 3-mm-thick sample. Although the epidermal fluorescence emission was reduced by the cell layers, the reduction width (approximately 150 A.U.) remained almost unchanged. Therefore, the autofluorescence changes on the tomato surface were attributed to the fluorophores in the cuticle.

The results of this study indicate that the tomato cuticle is the place where the surface autofluorescence changes during overripening. Till date, it has been widely accepted that the outermost layer of a plant material emits blue fluorescence when excited by UVA light[14] (coffee bean) and that the detached fruit cuticle absorbs UVA light[15] (apple) and emits autofluorescence (tomato).[8] However, tomato autofluorescence properties during overripening have not been investigated in detail. Thus, we investigated tomato autofluorescence properties during overripening as well as the influence of the removal of cell layers. To elucidate the novelty and significance of this work, we discuss the experimental validity and the possible explanation of this phenomenon.

To examine autofluorescence, the cuticle was enzymatically isolated. However, this methodology cannot always be applied to other fruits. Our preliminary experiments confirmed that the surface fluorescence characteristics remained unchanged even after the tomato was immersed in methanol or aqueous solutions (including the enzymatic cocktail), using a method similar to that proposed by Solovchenko and Merzlyak.[15] Therefore, the cuticular fluorophore of tomato was found to be insoluble. Therefore, the cuticle fluorescence properties seemed to be authentic and did not change our finding.

Possible fluorophores in the cuticle can be inferred based on the literature. Tomato cuticles contain fluorescent phenolic compounds,[16] which can be up to 1% of the total peel weight on a dry weight basis,[17,18] including flavonoid.[18] The autofluorescence of phenolics in the tomato fruit cuticle has also been mentioned in other studies,[1,7,19] although the authors did not measure the autofluorescence properties of isolated cuticles nor the *in situ*- autofluorescence properties. Further investigation is necessary for the identification of the fluorophores.

Apart from the fluorophores, other factors such as structure,[20,21] absorbers[15] in the cuticle, and cuticle hydration,[22–24] may also influence the autofluorescence properties. However, no structural changes were observed in the cuticle, epidermis (Fig. S3), and its autofluorescent width (Fig. 1). In addition, the visible-light-absorbing compounds, measured using the colorimeter, did not show any changes in the epidermis and cuticle; hence, their contributions seem to be small. As the water sorption of the cuticle is only a few percent,[12] the effect of hydration does not seem significant. According to the abovementioned discussion, we concluded that the changes in the tomato surface autofluorescence during overripening occurred within the cuticle.

Experimental section ‡

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Tomato plants (cultivar Momotaro, currently the most popular in Japan) were grown in a semi-open greenhouse at Ehime Research Institute of Agriculture, Forestry and Fisheries, Kumakogen-cho, Ehime, Japan. Red colour tomatoes (30 fruits in total) were harvested on July 27, 2019. After harvesting, the fruits were sent to Kyoto University. Upon arrival, the tomatoes were stored in a dark incubator (AS ONE Corp., Japan) at 25 ± 1 °C. The average relative humidity was maintained at 79 ± 5%. During storage, the fluorescence emission spectra of the tissues were measured. As a reference, the reflecting and fluorescence microscopic image and stained microscopic image of the cuticle were captured. For each of the three experiments conducted, three fruits were used at 3-day intervals up to day 13.

To investigate the distribution change in the fluorophore at both ends, the fluorescence microscopic images were obtained in a longitudinal section. The fruit was sectioned using a razor.[25] At least four sections were imaged per fruit from the blossom end and pedicel end without the considering the distal area and peduncle. The excitation source was a UV LED (CCS Inc., Japan) at 365 nm (with 10 nm width and an irradiance at the focus plane of 6 W m^{-2}) in a reflecting geometry. The source-to-sample distance was 40 mm. To filter out the reflected light, a long pass filter (50% cut at 430 nm) was attached in front of the camera, DFK 23U445 CCD (Imaging Source Asia Co., Ltd.). A macro lens (1×) was attached to the camera lens. This set-up provided a field of view of 2,830 × 2,120 μm^2 with a pixel size of 2.21 µm. The exposure time was set at 0.1–0.5 s to prevent saturation.

To investigate in which tissue the autofluorescence changes during overripening occurred, fluorescence emission spectra of four tissues (Table S1) were examined. A 5-mm-thick cylinder disc comprising the complete pericarp tissue was extracted using a tissue punch. Subsequently, a 3-mm-thick pericarp disc of 20 mm diameter was prepared using a razor. The 3-mm-thick pericarp included the outer epidermal cells, collenchyma cells, and parenchyma cells, but not the inner epidermal cells, as depicted in Fig. S2A. The tomato fruit peel (also termed as exocarp), including the outer epidermis and collenchyma cells, was manually detached from the remaining parenchyma tissue.[7,26] The measured peel thickness ranged from 86 µm to 486 µm (Table S1), in agreement with literature,[27] thus validating our protocol. Next, the epidermis was separated. Here, the epidermis is referred to as both the outermost epidermal cell layer and the inner one or two sub-epidermal cell layers,[28] resulting in one to three cell layers in total. This epidermis was manually delimited using the method proposed by Renaudin *et al.*[28] The epidermis thickness was 42–143 µm (Table S1), which coincides with that of one to three cell layers, thereby supporting our protocol.

A cuticle is an outermost cell wall of the epidermis, where cutin and waxes are densely accumulated. Based on this definition, its thickness ranged from approximately 25 µm to 40 µm.[4,29] The cuticle samples in this experiment ranged in thickness from 26 µm to 40 µm (Table S1). This good agreement validates our method. The cuticle isolation was conducted using the protocol proposed by Chatterjee *et al.*[30] First, a tomato peel of 30-mm diameter was detached using a tweezer, and gently washed with deionized water. Next, the peel was completely immersed in a 10-mL enzyme cocktail in a brown beaker and incubated at 31 °C for 24 h. The enzyme cocktail consisted of 1 w/v% pectinase (Sigma-Aldrich Co. LLC) and 0.01 w/v% cellulase (Tokyo Chemical Industry Co., Ltd., Japan) in a 50-mM pH 4.0 sodium acetate buffer with 65-mgL^{-1} NaN_3 (FUJIFILM Wako Pure Chemical Corp., Japan). After this incubation, the peel tissue was washed gently with deionized water again, eliminating the remaining cellular components. Thereafter, the isolated cuticle was air-dried at 30 °C in a dark oven. The samples were examined under an optical microscope to ensure that there were no epidermal cells attached to the cuticles. Fig. S4B shows the image of the isolated cuticle, which was yellow in colour, similar to that reported in previous studies,[7,30] compared with that of the epidermis (Fig. S4A). To estimate pigment accumulation in the four tissues, the colours of the tissues were measured using a colorimeter (TES135A plus, TES Co., Taiwan) under the D65 illuminant condition with a 2° observer angle.

The fluorescence emission spectra were measured using the spectrofluorometer FP-8300 (JASCO Corp., Japan). The spectral range of excitation and emission were 360 nm and 390–700 nm, respectively. The bandwidth was set as 5 nm for both slits. The photomultiplier tube sensitivity was set to prevent saturation. A response time of 50 ms and a scan speed of 5000 nm min^{-1} were set. The measurement was conducted for six biological replicates using the tissue isolated from three tomato fruits. Each tissue disc from the blossom end and the pedicel end was attached to the sample holder (20-mm-diameter quartz window). The incident and detection angles of light were 30° and 60°, respectively. The signal-to-noise ratio was 80–300.

Conclusions

In this study, we investigated the autofluorescence microscopic images at a longitudinal section and the fluorescence spectra of tomato surface tissues during overripening. We revealed that the tomato cuticle is the location where the autofluorescence changes occur up to day 13 (excitation at 360 nm). As autofluorescence can be captured using a commercial camera, this method is convenient for a low-cost implementation in the industry. Because the cuticle is universal in land plants, the quantification of its autofluorescence properties may help to standardize this type of techniques for other crops in future works.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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We acknowledge Prof. Garry John Piller for the useful comments on the paper. We are extremely grateful to Mr. Toshiki Masaoka and Mr. Kentaro Nishiwaki for providing the samples and experimental support, respectively. We also thank Mr. Shuhei Horiuchi, Mr. Ken Abamba Omwange, Mr. Katsuya Takenouchi, Mr. Yoshihisa Yamashige, and Mr. Khokan Kumar Saha for their technical support. We sincerely acknowledge and thank Matsushima Horticultural Development Foundation, Japan, and JSPS KAKENHI Grant Number 17J00302 for their financial supports.

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‡ Full experimental section and an appendix are available in the electronic supplementary information (ESI).

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