Association of fruit, pericarp, and epidermis traits with surface autofluorescence in green peppers

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We investigated the association of blue fluorescence (excitation at 365 nm) with the traits of the fruit, pericarp, and epidermis in green peppers. The fruits were manually classified into two groups based on fluorescence brightness. The dark fluorescence group showed the accumulation of blue-absorbing pigments and a thicker cuticular structure, suggesting epidermal development.

Introduction

Green pepper (*Capsicum annuum* L.) is one of the important vegetables that is consumed in many countries and is a rich source of vitamin C.[1] At the sorting facilities, colour and near-infrared (NIR) imaging has been used to inspect the size, shape, and surface defects. More recently, UV-induced fluorescence imaging has been implemented by several research groups, including our group, because it can highlight the surface defects, as demonstrated by Fatchurrahman *et al.*[2] The authors showed that the potential excitation wavelength was 365 nm. However, at the sorting facilities, there are large variations in the autofluorescence characteristics of green peppers even without defects. Thus, some green peppers are misclassified as abnormal. This prevents the implementation of this technology in this field. Therefore, the understanding of the autofluorescence variation among green peppers is necessary.

The surface autofluorescence characteristics can be understood in terms of the traits of the epidermis and pericarp (*i.e.*, epidermis and inner cells) that form the whole fruit. Ramos and Lagorio,[3] Novo *et al.*,[4] and Calvo *et al.*[5] characterised the fluorescence characteristics through the association with the surface tissues for apples, kiwis, and eggplants. Stelzner *et al.*[6] reported the autofluorescence characteristics of sunflower leaves by the association with the surface tissues through microscopic fluorescence imaging. For green peppers, several researchers have investigated the surface and inner autofluorescence properties.[2,7–12] However, the association of the surface autofluorescence characteristics of green peppers with the traits of the fruit, pericarp, and epidermis has not been investigated yet.

In this study, we investigated the association of the traits of the fruits with autofluorescence in green peppers. First, we explored the region where fluorophores exist to determine the appropriate tissue for exploring the traits. In this step, the fluorophore regions were investigated by microscopic fluorescence imaging (excitation at 365 nm) at the cross section and fluorescence spectroscopy was applied for the surface tissues (*i.e.*, pericarp and epidermis). Next, we correlated the traits at the scales of the fruit, pericarp, and epidermis with the surface autofluorescence characteristics using statistical analysis. Finally, we discussed the biological meaning of surface autofluorescence in association with the traits and the implications of surface autofluorescence as an index of quality.

Results

First, we manually divided the green peppers into two groups based on the brightness of surface autofluorescence under excitation at 365 nm. Fig. 1A shows the images of the two groups, namely, bright and dark groups. The intensity of blue

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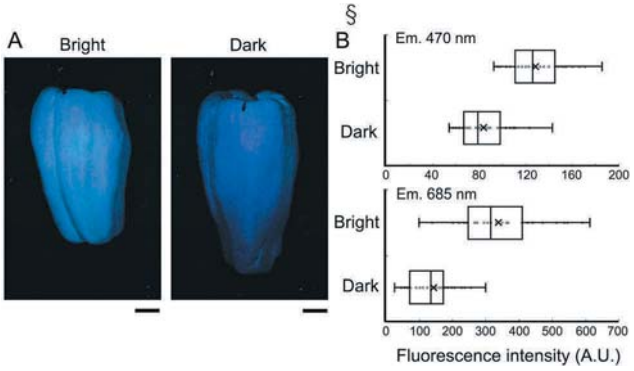

Fig. 1 (A) Fluorescence images of green peppers (excitation at 365 nm). Bar = 10 mm. (B) Fluorescence intensities at emissions of 470 and 685 nm (excitation at 365 nm). The two groups are shown *viz*. bright (n = 39) and dark fluorescence (n = 40). The image depends on the RGB sensitivity. § The intensities were calculated from the fluorescence spectra of the fresh pericarp (Fig. S2†).

fluorescence was lower for the dark group. However, the corresponding colour photographs were similar between the two groups, as shown in Fig. S1.† To confirm these trends quantitatively, the fluorescence spectra of each fruit were obtained using a fluorophotometer. Fig. S2† shows the spectra of the green pepper pericarp. The excitation wavelength was 365 nm, which was the same as that for the imaging. The bright group showed three peaks at 410, 470, and 685 nm. In contrast, the dark group showed only two peaks at 410 and 685 nm (because the peak at 470 nm was not prominent), and the intensity of the peak at 685 nm was lower than that of the bright group. Therefore, the group difference was characterised by the two intensities at 470 and 685 nm (Fig. 1B).

Before investigating the relationship between the surface autofluorescence characteristics and the traits, we used the cross-sectional fluorescence microscopic images of green peppers (Fig. 2) to investigate the regions where the fluorophores exist. Both the bright and dark groups showed bright emission along the surface. Furthermore, the emission near the surface was highly intense such that it was partially saturated when imaging was conducted including a weak emission in inner tissues. The inner tissues showed red fluorescence, mainly from chlorophyll.[10,11] The red fluorescence was observed over 200 μm in the depth direction similar to that of other crops.[13,14] This suggests that chlorophyll emission occurred from tissues including the epidermis[15] and from other cells below the epidermis.[16] To investigate the tissues that emit blue fluorescence, we measured the thickness of the fluorescence width located near the surface. The width did not exceed 50 μm for both groups. This indicated that the fluorophores exist within the outermost single cell layer, which is referred to as the epidermis.[17]

To investigate whether surface autofluorescence can be ascribed to the epidermis, we measured its fluorescence spectra by isolating the tissue. We compared the spectra with those of the fresh pericarp. It is to be noted that the epidermal membrane was isolated and subsequently air-dried.[18,19] In this study, we have termed this sample as the dried epidermis. The fluorescence emission spectra exhibited two peaks centred at 470 and 685 nm (Fig. 3A and S3†). The peak centred at 470 nm included minor shoulders at 410 and 430 nm. The emission centred at 470 nm corresponded to the blue emission observed non-destructively (Fig. 1A). The peak at 685 nm was associated with the red fluorescence in the microscopic images, which was emitted from the outermost epidermal cells (Fig. 2) as described in a previous study.[15]

Fig. 3B shows the fluorescence intensities of the epidermis obtained from the fluorescence emission spectra shown in Fig. 3A. The two fluorescence peaks at emissions of 470 nm

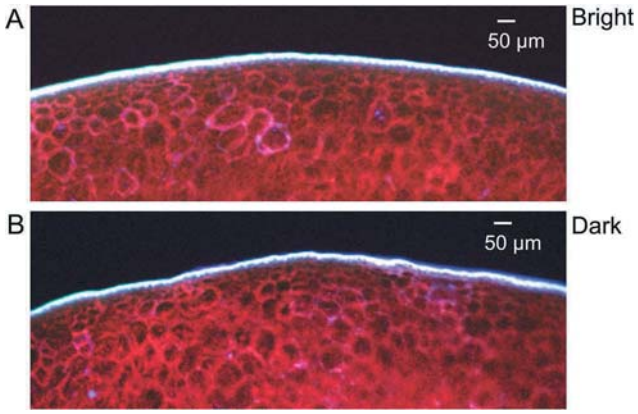

Fig. 2 Fluorescence microscopic images of green peppers at the cross-section. (A) Bright-fluorescent and (B) dark-fluorescent fruits. The black–white interface observed on the upper side indicates the surface of the green pepper and its flesh below. The image depends on the RGB sensitivity.

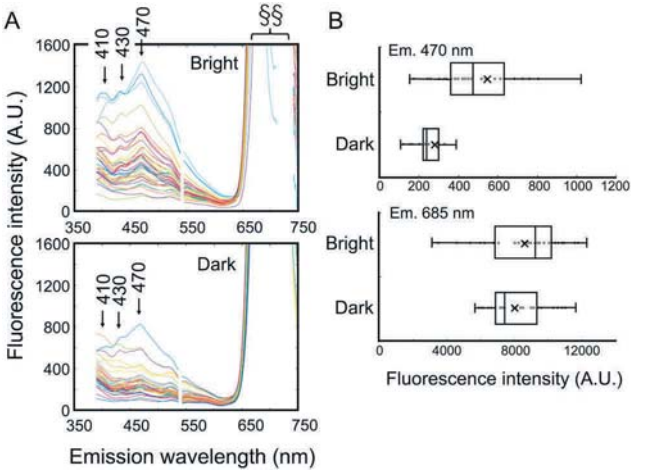

Fig. 3 (A) Fluorescence emission spectra of the dried epidermal membrane of green pepper fruits (excitation at 365 nm). (B) Fluorescence intensities at emissions of 470 and 685 nm (excitation at 365 nm). The spectra at around 550 nm were removed due to distortions in the device. The two groups are shown: bright (n = 39) and dark fluorescence (n = 40). §§ A rescaled figure for the peaks at 685 nm is presented in Fig. S3.†

and 685 nm were larger than those of the pericarp. We inferred that the increase in the fluorescence intensity for the epidermis was caused by at least two factors, *i.e.*, dehydration[20] and the increase in the fluorophore concentration by drying it in a smaller volume. By isolating and drying the epidermis, the average thickness changed from 33.1 to 23.6 μm and 40.0 to 23.6 μm for the blue and dark fluorescence groups, respectively. Despite such differences between the intact and dried epidermis, the fluorescence intensities of the dried epidermis showed the same tendency as those of the intact pericarp at 470 nm (Fig. 1B). The fluorescence intensities were lower for the dark group. The fluorophores that characterised the variation in the autofluorescence were located in the epidermis.

We have recognised the epidermis as an important tissue to correlate its traits with the autofluorescence characteristics. Therefore, we further investigated the relationship between the epidermal traits and surface autofluorescence characteristics. The colour of the epidermis is one of the important traits because it is related with epidermal development.[21] Fig. 4A shows the photographs of the dried epidermal membranes. For both groups, the epidermal membranes were yellow, as reported previously.[21] However, the yellow colour of the dark group was darker. To investigate the colour difference, we measured the RGB colour values by using a colour meter and converted the values into absorption units (*i.e.*, the logarithmic values of the reciprocals of the diffuse reflectance; 600, 550, and 450 nm for the RGB center wavelengths, respectively). In general, a sample with yellow colour absorbs blue light (a complementary colour). Thus, the absorption of blue light is shown in Fig. 4B. In the dark group, the absorption of blue light was stronger than that in the bright group. This difference implies a high accumulation of blue-absorbing pigments within the epidermis for the dark group.

The structure of the epidermis is also important because the epidermal thickness is related with fruit development[22] and the shelf-life.[23] Fig. 5A shows the cuticular structure of the epidermis for the two groups. The cuticle, in which the cutin, waxes,[24] and phenolics[7] were densely accumulated, was stained red. For the bright group, the cuticular structure of the epidermis was band-shaped and it did not uniformly cover the inner cells. This strongly suggests that the cuticle was fragile and immature. In contrast, the dark group showed a uniform structure of the outer wall of the cuticle. A quarter of the fruits showed the cuticular thickness greater than 31 μm, as shown in Fig. 5B. The group also showed a cuticle deposit within the epidermal cells, which is also called an anticlinal peg[25] or

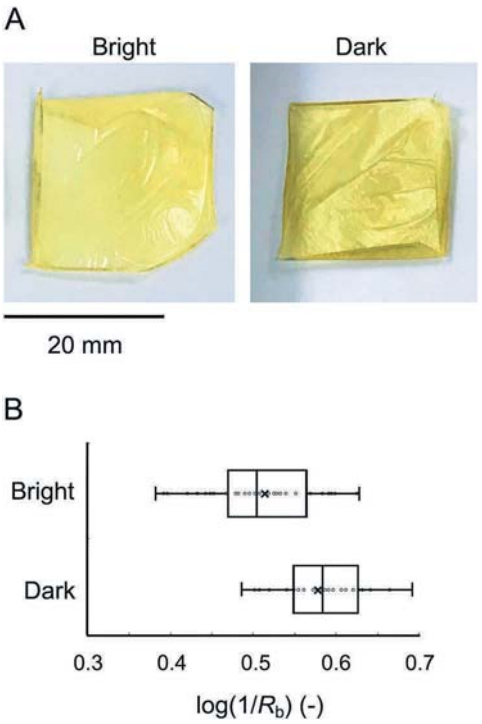

Fig. 4 (A) Photographs of the isolated and dried epidermal membrane of green pepper fruits. (B) Absorption unit (*i.e.*, the logarithmic value of the reciprocal of the diffuse reflectance) of blue light (approximately at 450 nm) for the two groups of green peppers: bright (n = 39) and dark fluorescence (n = 40).

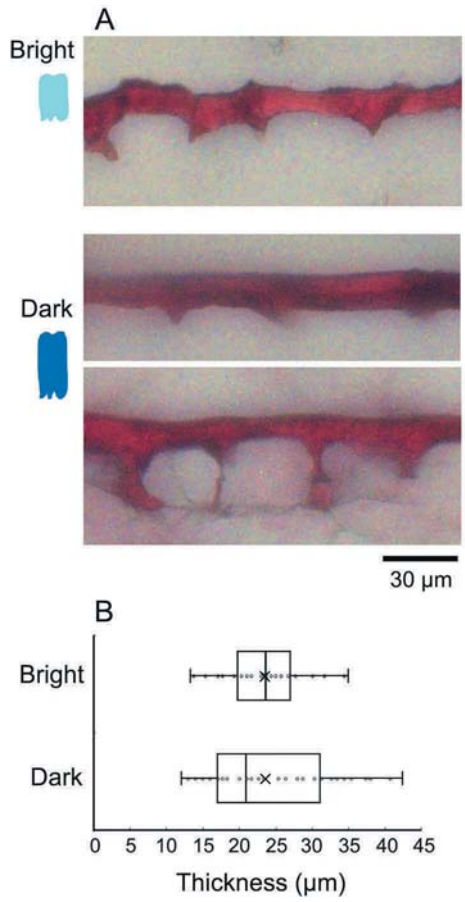

Fig. 5 (A) Cuticle stained microscopic images of the epidermis in green pepper fruits for the two groups: bright and dark fluorescence. The cuticle is stained red. (B) Thickness of the dried epidermis. The two groups are shown: bright (n = 39) and dark fluorescence (n = 40). Two cuticular patterns are observed for the dark groups.

invagination.[26] The variation in the thickness of the dried epidermis agrees with our microscopic observation shown in Fig. 5A. The dark group showed a relatively greater thickness. The ranges were 13–35 μm and 12–42 μm for the bright and dark groups, respectively. This suggests that the dark fluorescent green peppers tend to have a thicker dried epidermis and cuticle, accompanied by the uniform thickness of the outer wall.

We further explored the association of other traits (*e.g.*, size, colour, and thickness of tissues) with the surface autofluorescence characteristics using statistical analysis. The 24 traits used in this analysis are listed in Table S1 in the ESI.† The scatter plot was not suitable as the fluorescence intensities are affected by many factors. We used partial least squares-discriminant analysis (PLS-DA) to visualise the important traits related with surface autofluorescence. Using PLS-DA, we have also discussed the biological meaning of surface autofluorescence and its implications as an index of quality.

Fig. S4† shows the 1st and 2nd PLS scores, which explain 60% and 26% of the variance of the 24 traits (86% in total). As shown in the plot, the differences between the two fluorescence groups are characterised by the 1st PLS component. Conversely, the 2nd and 3rd PLS scores are not significantly related to the differences between the two groups (Fig. S4 and S5A†).

Fig. 6 shows the corresponding loading plot of the 1st and 2nd PLS components. Each plot indicated the relative contribution vector of the trait to the 1st and 2nd PLS components. In particular, we focus on the contribution of the traits to the 1st PLS component because it distinguishes the two groups. The 1st axis value corresponds to the 1st PLS component. The positive loading indicates that the trait is higher for the dark group. The 1st loadings of the fluorescence intensities of the epidermis at 410 and 470 nm are −6.90 and −7.31, respectively (F410, 470_epi). This indicates that the dark group is characterised by the lower intensities of fluorescence emission from the epidermis at 410 and 470 nm. This ensures the high contribution of the epidermal fluorescence to the surface autofluorescence characteristics. In contrast, the light absorption properties of the fruit and epidermis are located in the positive direction of the 1st PLS loading (r, g, b_epi). In particular, the blue absorption of the dried epidermis shows the highest 1st loading of 7.22 among other traits. This is consistent with the obtained results shown in Fig. 4. The fruit length also shows a high 1st PLS loading of 5.53. This is also supported by the descriptive statistics values as shown in Fig. S6.† As the growth stage of green peppers can be defined by the fruit length,[22] we can associate the fluorescence darkness with fruit growth. However, there is no strong correlation between the fruit length and the fluorescence intensities at 410 and 470 nm (r = −0.32 and −0.41, respectively). This suggests that the accumulation of the fluorophores and yellow pigments[21] (*e.g.*, phenolics[7]) is not only related with fruit growth[22] but also related with environmental factors such as humidity, light, temperature and pathogens.[27] Thus, it is reasonable that the fluorescence intensities do not show a high correlation directly with the fruit length. However, the results show that the colour and cuticular structure of the epidermis can potentially be evaluated by the surface fluorescence of green peppers.

Fig. 6 also shows the association of the moisture content and firmness with the autofluorescence characteristics. These are important qualities as they can change depending on the storage condition and site.[28,29] The moisture content shows a low correlation with the fluorescence groups (−1.33 for the 1st loading). Meanwhile, the firmness shows a moderate correlation with the groups, depending on the region of the fruit (firm_p and firm_b for pedicel and blossom ends, respectively). The firmness at the blossom end is correlated with the two groups (−4.00 for the 1st loading), while the firmness at the pedicel end shows a comparatively lower correlation with the groups (−0.26 for the 1st loading). The lower firmness at the blossom end for the dark group may be related to the longer shape of the fruit for that group. However, there is no clear association between the fluorescence characteristics and many traits except for the colour and cuticular structure of the epidermis.

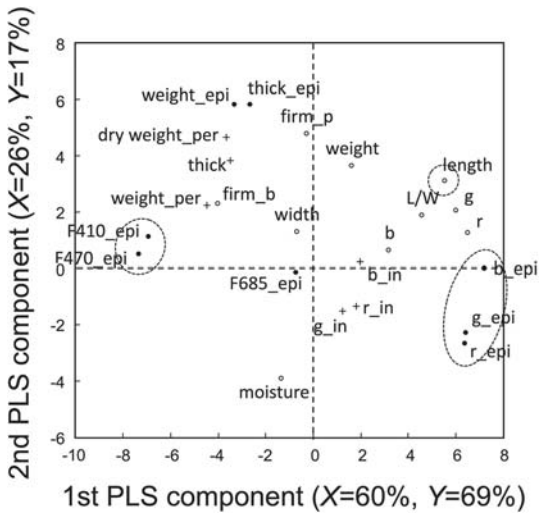

Fig. 6 PLS-DA loading plot. Component 1 *vs.* 2. The percentage in the axis shows the explanation power of each PLS component for the *X*- and *Y*-variables (24 traits and binary vectors of the groups (−1 and 1), respectively). The positive value of the 1st loading is related with the dark group. The abbreviations F410, F470, and F685 stand for the fluorescence of the dried epidermis (epi) at 410, 470, and 685 nm, respectively (excitation at 365 nm). r, g, and b indicate the absorption of red, green, and blue light, respectively, at the fruit surface, inner (in) pericarp, and dried epidermis (epi). The other variables can be referred to in Table S1 in the ESI.† The open circle, cross, and filled circle indicate the traits of the fruit, pericarp, and epidermis, respectively.

Conclusions

In this study, we investigated the association of green pepper autofluorescence with the traits of the fruit, the pericarp, and the epidermis (excitation at 365 nm). First, we investigated the regions where the fluorophores are located to determine the appropriate tissue for exploring the traits. By using fluo-

rescence microscopic imaging (excitation at 365 nm) and a fluorophotometer, we revealed that the epidermis plays a significant role in characterising the blue fluorescence on the fruit surface (emissions at 410 and 470 nm). Subsequently, we correlated the two fluorescence groups with the traits of the epidermis, pericarp, and fruit using PLS-DA analysis. The dark fluorescence group showed a high accumulation of blue-absorbing pigments within the epidermis, a thicker cuticular structure accompanied by the uniform thickness of the outer wall, and a longer fruit length. These results suggest that surface autofluorescence could be an indicator of epidermal development, as well as provides a means for the non-destructive assessment of the postharvest quality because the epidermal development is essential for a longer shelf life.

Experimental section

The full Experimental section is available in the ESI.†

Author contributions

NK and FRM supervised the study. KK and DFAR designed the experiments. HN and KN provided the samples. KK, KAO, AK, and AMO conducted the experiments. KK and KAO analysed the data. DFAR provided the experimental tools. KK wrote the original draft. KK, KAO, DFAR, and AK reviewed and edited the manuscript.

Conflicts of interest

There are no conflicts to declare.

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