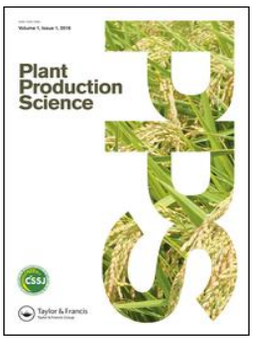

Plant Production Science

ISSN: 1343-943X (Print) 1349-1008 (Online) Journal homepage: https://www.tandfonline.com/loi/tpps20

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To cite this article: Akira Yano, Masato Takakusagi, Kazushi Oikawa, Shinsuke Nakajo & Takashi Sugawara (2017) Xanthophyll levels in foxtail millet grains according to variety and harvesting time, Plant Production Science, 20:1, 136-143, DOI: 10.1080/1343943X.2016.1246347

To link to this article: https://doi.org/10.1080/1343943X.2016.1246347

PLANT PRODUCTION SCIENCE, 2017
VOL. 20, NO. 1, 136–143
http://dx.doi.org/10.1080/1343943X.2016.1246347

REGULAR PAPER

OPEN ACCESS

Xanthophyll levels in foxtail millet grains according to variety and harvesting time

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ABSTRACT

Foxtail millet grains usually contain carotenoids, which are yellow pigments that are important for human health. Yellow grains are preferred by distributors and consumers, and special cultivars and cultivation methods are desired for a stable supply of yellow millet. We investigated the level of pigment fluctuation in several foxtail millet accessions, including the yellow grain cultivar 'Yuikogane' from Iwate Prefecture, using high-performance liquid chromatography. Most yellow grains primarily contained xanthophylls, including approximately 1 mg/100 g lutein and 0.2 mg/100 g zeaxanthin. These pigments were rare in the bran and grain husks but were readily detected in polished grains, indicating that xanthophylls accumulate in the endosperm. We examined 'Yuikogane' to investigate the relationship between xanthophyll accumulation and grain ripening. During the ripening stage, xanthophyll levels gradually increased, but they rapidly decreased in response to over-ripening. Xanthophyll accumulation was estimated using a colorimetric assay of yellow pigmentation, which could be a useful method for determining the proper harvesting time for foxtail millet.

ARTICLE HISTORY
Received 4 July 2016
Revised 21 September 2016
Accepted 4 October 2016

KEYWORDS
Foxtail millet; xanthophyll; lutein; zeaxanthin; colorimeter; harvest time

CLASSIFICATION
Crop Physiology

Introduction

Foxtail millet (*Setaria italica* (L.) P. Beauv.) is an ancient crop that remains agronomically important, especially in Asia (Barton et al., 2009; Lu et al., 2009). Millet consumption has declined as rice consumption has increased; however, millet remains a daily dietary staple for many people in Asia (Dixit et al., 2011; Yang et al., 2012). Millet contains many more active nutritional compounds than white rice, including dietary fiber (Montonen et al., 2003), proteins, vitamins, minerals, and important phytochemicals such as phytic acid (Ravindran, 1991) and ferulic acid (Mattila et al., 2005). Millet consumption is thought to reduce the risk of chronic disease development (Das et al., 2012; Dixit et al., 2011; Rao et al., 2011).

The major active ingredients of millet are thought to be antioxidants and dietary fiber (Dykes & Rooney, 2007; Rio et al., 2013). Pigments and anthocyanins in black, blue, pink, purple, and red cereal grains are believed to have antioxidant activities (Abdel-Al et al., 2006). Yellow and red pigments in millet are also considered to function as antioxidants. Asharani et al. (2010) reported that carotenoids in Indian finger millet varieties (including several foxtail and proso millet varieties) function as antioxidants, but they did not identify the specific carotenoid compounds. McGraw et al. (2001) identified the xanthophyll pigments lutein and zeaxanthin in millets by high-performance liquid chromatography-photodiode array detector (HPLC-PAD) analysis. However, they did not indicate the specific names of the millets investigated; instead, they used common names, such as bird feed, red and white millets, for the proso millet varieties. Zhang et al. (2014) detected lutein and zeaxanthin in proso millet grains. Lutein and zeaxanthin are predicted to contribute to the yellow and red colors of millet grains, respectively. The major yellow pigments of foxtail millet (Chinese cultivars) have recently been reported to be lutein and zeaxanthin (Shen et al., 2015).

In this study, we analyzed the yellow pigment content of several foxtail millets (Japanese cultivars and landraces) to identify major yellow pigments and determine their levels in unpolished grains. The cultivar 'Yuikogane,' bearing typical yellow grains, was developed at Kenpoku agricultural institute by selecting cultivars from a cross between two landraces of Iwate prefecture, namely, 'Nisatai-zairai' (female), and 'Otsuchi 10' (male) (Nakajo, 2015). It inherited a bright yellowish endosperm, big grain size and a thick stem from the maternal line, and a glutinous endosperm from the paternal line. Another cultivar 'Shinanotsubuhime,'

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Supplemental data for this article can be accessed here. [http://dx.doi.org/10.1080/1343943X.2016.1246347]

also bearing yellow nonglutinous grains, was developed in Nagano prefecture.

The acreage under 'Yuikogane' cultivation has increased in recent years and new cultivation methods to increase the supply of yellow grains are required. We investigated the time course of xanthophyll accumulation during ripening and investigated the best harvesting time for the acquisition of healthy yellow grains.

Materials and methods

Reagents and chemicals

All solvents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO, U.S.A.), and were of analytical grade or high-performance liquid chromatography (HPLC) grade. Lutein and zeaxanthin were obtained from ChromaDex, Inc. (Irvine, CA, U.S.A.) and dissolved in ethanol to a concentration of 1 mg/ml, and stored at −35 °C. These stock solutions were diluted with ethanol to generate standard solutions.

Plant materials

Foxtail millets were cultivated at Kenpoku Agricultural Research Institute (Iwate Prefecture, Japan) and harvested in autumn of 2015. Two cultivars, Yuikogane (registration number 25234) and Shinanotsubuhime (reg. no. 21738), and 20 other landraces used in this study are listed in Table 1. These accessions were selected from the collection at the Kenpoku Agricultural Research Institute. Dried seeds, harvested at maturity, were threshed and stored under dry, dark conditions at 15 °C in a seed storage room. Whole foxtail millet grains were prepared using a husking machine (FC2 K, Otake Seisakusyo Co., Ltd., Aichi, Japan) and stored at −20 °C before analysis. Whole grains were polished to remove the outer aleurone layer and embryo (bran) using a polishing machine (CX-30 K, MARUMASU KIKAI Co., Toyama, Japan). Grains and grain fragments were observed using a digital stereo-microscope (DSX500, Olympus KK, Shinjuku, Tokyo, Japan).

Grain extracts and sample processing

Samples (~10 g) of whole grain, polished grain, bran, and husks were crushed to a powder using a cutter mill (IFM-620DG, Iwatani Corporation, Tokyo, Japan). Methanol (10 ml) containing 3% (w/v) pyrogallol was added to 0.5–1.0 g of ground powder. The pigments were extracted by sonication for 2 min following 30 min shaking at 50 °C (Cool shaker, ASONE Co. Ltd. Tokyo, Japan). The supernatant extract was collected and filtrated through a 0.45-µm Minisart filter (Sartorius Japan K.K., Tokyo, Japan) before analysis.

HPLC-PAD analysis

To identify the major pigments in the extracts, 20-µl samples were separated using an analytical column (Carotenoid C30, 4.6 × 250 mm, 5-µm particle; YMC Co., Ltd, Kyoto, Japan) with a flow rate of 1.0 ml/min at 40 °C,

Table 1. Lutein and zeaxanthin contents in unpolished grains of *Setaria italica* (L.) P. Beauv. accessions. Millets were harvested from fields at Kenpoku Agricultural Institute. The seeds were husked, and the unpolished grains were subjected to methanol extraction and HPLC-PAD analysis. Contents are expressed as the mean ± SD.

Accession name	L + Z	Lutein		Zeaxanthin		b*	Remarks
Toranoo	2.489	1.890	± 0.022	0.599	± 0.086	36.89	
Nisatai-zairai	2.247	1.875	± 0.058	0.372	± 0.040	37.61	
Shinanotsubuhime	2.167	1.828	± 0.108	0.339	± 0.031	35.85	21,738*
Ohfusa awa	2.099	1.712	± 0.010	0.387	± 0.056	39.34	
Yuikogane	1.668	1.259	± 0.091	0.410	± 0.016	39.74	25,234*
88-9-18-34	1.665	1.342	± 0.042	0.323	± 0.011	39.38	71,631**
Taira17	1.523	1.201	± 0.059	0.322	± 0.007	37.12	
Takachiho	1.438	1.081	± 0.094	0.356	± 0.030	39.75	
Okayama	1.200	0.912	± 0.023	0.287	± 0.009	36.18	
Kitsurinsyou	1.106	0.905	± 0.052	0.201	± 0.012	34.38	
China derived	0.869	0.654	± 0.078	0.216	± 0.026	33.44	
Tohno7	0.412	0.319	± 0.065	0.092	± 0.007	20.93***	
Fukuoka	0.404	0.334	± 0.073	0.070	± 0.011	30.86	
Iwate zairai6	0.391	0.327	± 0.081	0.064	± 0.005	30.65	
Shina Ooawa	0.360	0.306	± 0.084	0.053	± 0.007	30.39	25,114**
Takizawa39	0.330	0.300	± 0.037	0.030	± 0.030	30.56	
Yamada15	0.166	0.157	± 0.092	0.010	± 0.014	26.11	
Yukiyamochi	0.161	0.147	± 0.091	0.014	± 0.017	26.63	53,582**
Ohtsuchi10	0.149	0.149	± 0.087	ND	± 0.007	27.82	
Yamada4	0.122	0.122	± 0.072	ND	± 0.006	26.12	
Ninohe14	0.121	0.121	± 0.074	ND	± 0.006	25.43	
Yatsufusa awa	0.119	0.119	± 0.088	ND	± 0.004	25.36	

*Registration numbers of plant varieties at the Ministry of Agriculture, Forestry, and Fisheries;
**Accession numbers in the Genebank Project of the National Agriculture and Food Research Organization;
***The greyish color of the grains might have reduced the b* value.

and absorbance at 200–600 nm was monitored (Waters 600 Quad Pump and Controller, Waters 717 Plus Automatic Sampler and Waters 996 Photodiode Array Detector; Waters Corp., Milford, MA, U.S.A.). A binary gradient was used for elution, with mobile phases of 50% acetonitrile, 45% methanol, plus 5% distilled water (Solvent A), and 95% isopropanol plus 5% distilled water (Solvent B). Optimized pigment separation was achieved using a gradient of 15–25% Solvent B from 0–30 min, and the same gradient was also used for LC-MS/MS analysis (Supplemental Methods). For routine analysis, 20 µl of extract was injected into a HPLC-PAD system (Alliance 2960 HPLC system, Waters Corporation, Milford, MA, U.S.A.). Extracts were separated using an analytical column (Develosil XG C-30 M, 4.6 × 250 mm, 5-µm particle; Nomura Chemical Co., Ltd, Aichi pref., Japan) with a flow rate of 1.0 ml/min at 40 °C, and absorbance at 400–500 nm was monitored. Pigment separation was achieved with acetonitrile/methanol isocratic mobile phase (65:35, v/v).

Colorimetry analysis

Grains, harvested in maturated stage, dried and unhusked, were spread in a Petri dish, and color was measured using a colorimeter in L*a*b* measurement mode according to the manufactures' manual (CR-410, KONICA MINOLTA JAPAN, INC., Tokyo, Japan).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of three measurements. Student's *t*-test was computed using Excel software. A significant difference was defined as $p < 0.05$. Linear regression analysis was also done using Excel software.

Results

Identification of the yellow pigments of foxtail millet

To identify the major yellow pigments of foxtail millet grains, we analyzed several accessions, including typical yellow- and white-grain cultivars and landraces, by HPLC-PAD. Two major yellow pigments with absorbance at 450 nm were resolved in yellow grain samples (Figure 1(a), Yuikogane). The standards were examined using absorbance at 450 nm, and the retention times of the lutein and the zeaxanthin standard peaks coincided well with those of 'Yuikogane' extract Peak 1 and Peak 2.

We compared the spectral profiles of the peaks (Figure 1(b)). The absorbance peaks were resolved for the lutein standard at 445 and 472 nm, which was also observed for Peak 1. In addition, the absorbance peaks were resolved for the zeaxanthin standard at 450 and 478 nm, which was also observed for Peak 2 (at 449 and 473 nm).

We analyzed the two main peaks of 'Yuikogane' using a triple-quad LC-MS/MS system (Figure S1). To reduce the background ion noise from the matrix, we fractionated the yellow pigments by HPLC and diluted pigment-rich fractions with methanol. We then subjected the diluted fractions to LC-MS/MS analysis and compared the mass spectra with those of the lutein and zeaxanthin standards. The Q3 scan data, including the parental ions of pigment molecules and small ions of collapsed molecules, are shown in Figure S1. The molecular weight of lutein and zeaxanthin is 568.87114 (PubChem, NCBI database). The major ion in the lutein standard had an *m/z* of 551.7, indicating that the ion corresponded to $[M + H - H_2O]$. The major ion in the 'Yuikogane' pigments (corresponding to Peak 1 in Figure 1(a)) also had an *m/z* of 551.7. The major ions in the zeaxanthin standard had *m/z* values of 551.7 and 569.6, which were estimated to correspond to $[M + H - H_2O]$ and $[M + H]$, respectively. The spectral profile of 'Yuikogane' Peak 2 was relatively unstable. The Peak 2 fraction might contain contaminants inhibitors of the pigment ionization, because the ion peak (*m/z* = 569.6), estimated to be $[M + H]$, was weaker than zeaxanthin standard.

Quantification of lutein and zeaxanthin in foxtail millet cultivars

The two major yellow pigments of 'Yuikogane' were identified as lutein and zeaxanthin based on the HPLC-PAD and LC-MS/MS data. We quantified the amounts of lutein and zeaxanthin in unpolished grains of several cultivars and landraces by HPLC-PAD analysis (Table 1). Foxtail millets were grown and harvested at the Kenpoku Agricultural Research Institute. Unpolished grains contained 0.119–1.89 mg lutein per 100 g of grain and 0–0.599-mg zeaxanthin per 100 g of grain. The amount of lutein was correlated with the amount of zeaxanthin (correlation coefficient is 0.945), and the most xanthophyll-rich cultivar, Toranoo, contained 2.489 mg/ 100 g xanthophylls in the unpolished grains. By contrast, several foxtail millets contained approximately 0.1 mg/ 100 g of lutein and zeaxanthin levels below the detection limit.

Localization of lutein and zeaxanthin in foxtail millet

We examined the localization of lutein and zeaxanthin in foxtail millet. The relative amounts of lutein and zeaxanthin in 100 g of polished grain, bran, whole grain (bran and polished grain), and husks are shown in Figure 2(a). Images of unpolished and polished grains from 'Ohtsuchi10' and 'Yuikogane' are shown in Figure 2(b). Unpolished

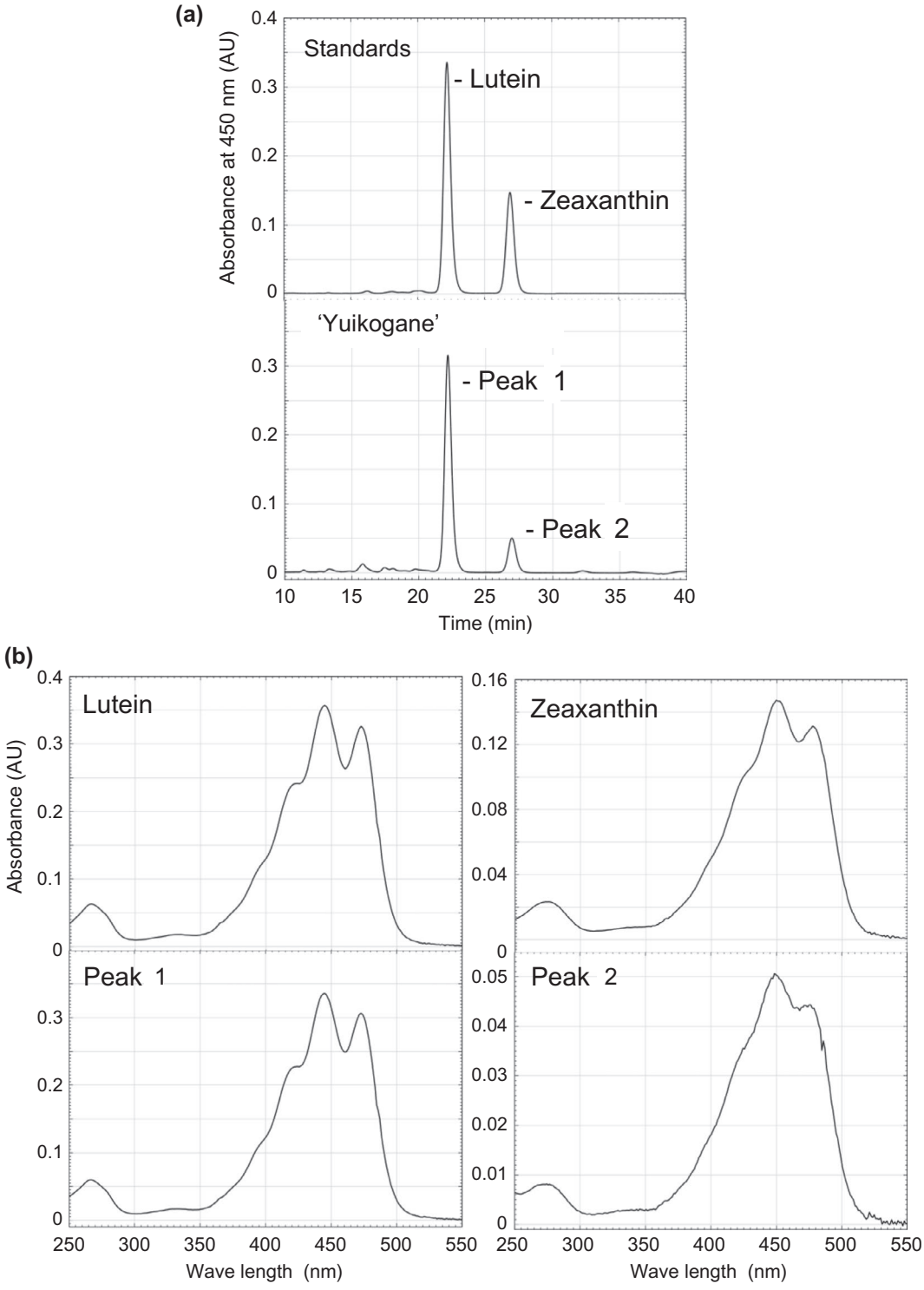

Figure 1. HPLC-PAD spectral profiles of the major yellow pigments in foxtail millet grains compared with those of lutein and zeaxanthin standards. (a) Retention times of peaks 1 and 2 of cultivar 'Yuikogane.' (b, c) Absorption spectra of each peak for yellow pigments in cultivar 'Yuikogane' and in the lutein and zeaxanthin standards.

'Ohtsuchi10' and 'Yuikogane' grains were white and yellow, respectively. After polishing, the endosperm of 'Yuikogane' was yellow (Figure 2(b)). Unpolished and crushed grain fragments of 'Ohtsuchi10' and 'Yuikogane' were also white and yellow, respectively (Figure 2(c)).

Correlation between the amounts of xanthophyll and the yellow color of foxtail millet grains

The grain colors of various foxtail millets, as shown in Table 1 were analyzed by colorimetry. The b* value reflects the

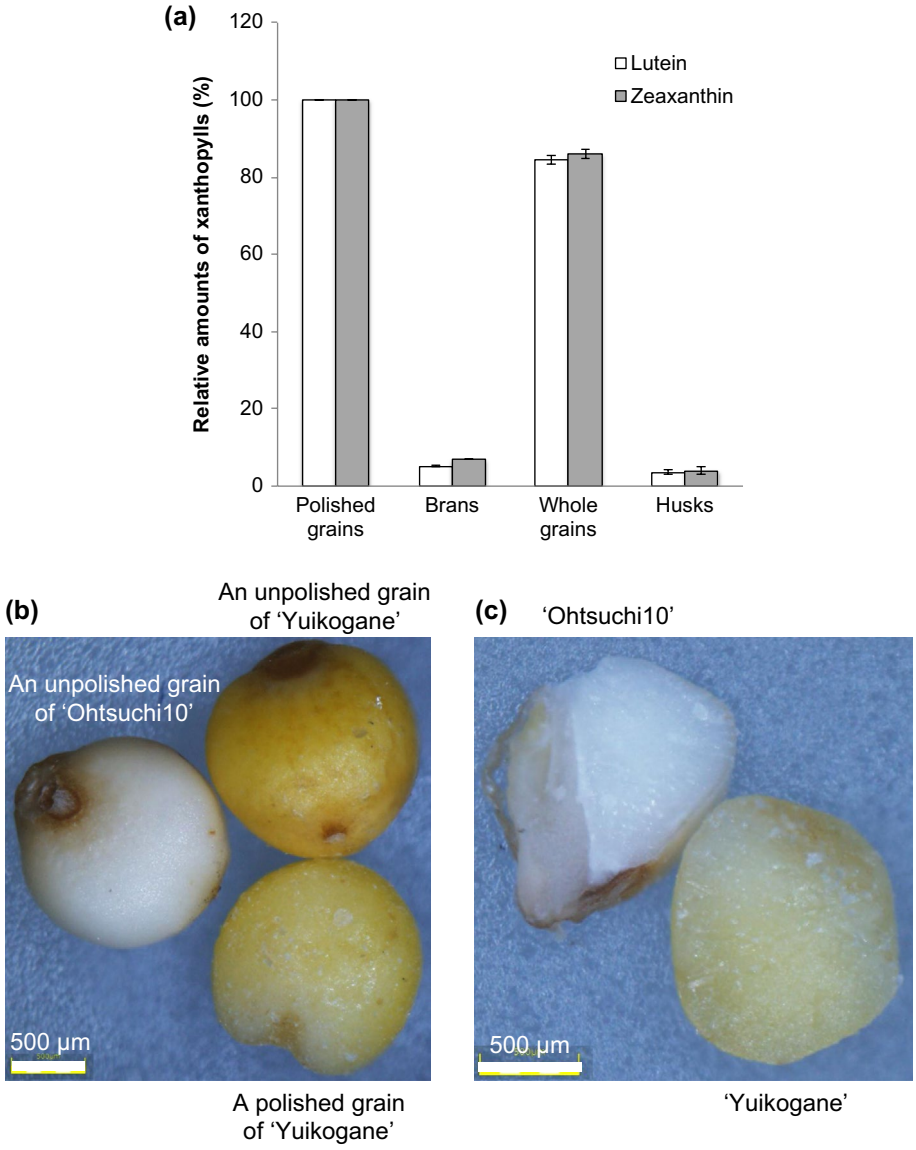

Figure 2. Localization of xanthophyll accumulation in 'Yuikogane.' (a) Seeds were husked, and whole unpolished grains were polished and separated into bran and polished grains. All fractions were extracted with methanol and subjected to HPLC-PAD analysis ($n = 3$). Xanthophyll contents (mg/100 g sample) were measured (± SD) and normalized with respect to the xanthophyll content in polished grains, which was set to 100%. (b) Unpolished grains of 'Yuikogane' and 'Ohtsuchi10' and a polished grain of 'Yuikogane' observed under a stereo microscope. The bran and embryo were removed from the polished 'Yuikogane' grain. (c) Unpolished and crushed grain fragments of 'Yuikogane' and 'Ohtsuchi10' observed under a stereo microscope.

strength of the yellow color. The scatter plot of b* values and xanthophyll concentrations clearly indicate that these values are positively correlated (Figure 3(a) and (b). The yellow color (b*) can therefore be used as an indicator of xanthophyll levels in grains. However, foxtail millets with the highest levels of lutein ('Toranoo,' 'Nisatai-Zairai,' and 'Shinanotsubuhime') had a lower b* value than those estimated by linear regression.

Grain color and xanthophyll accumulation during ripening

We measured xanthophyll accumulation and unpolished grain color during the ripening stage in 'Yuikogane.' Grain samples were collected every 5 days beginning at 22 days after the heading date. Yellow grain color was measured by colorimetry, and xanthophyll levels were measured by HPLC. We investigated the relationship between b* and lutein concentration in the 'Yuikogane' samples (Figure 4(a)), as these grain samples had the same hue but a higher correlation between b* and lutein levels than several other foxtail millets (Figure 3). As shown in Figure 4(b), the changes in lutein concentrations and b* value of unpolished 'Yuikogane 'grains exhibited similar trends. Lutein levels increased until the accumulated temperature from heading reached approximately 850 °C and gradually decreased until 1150 °C. Over 1150 °C, the lutein content suddenly decreased. Above an accumulated temperature

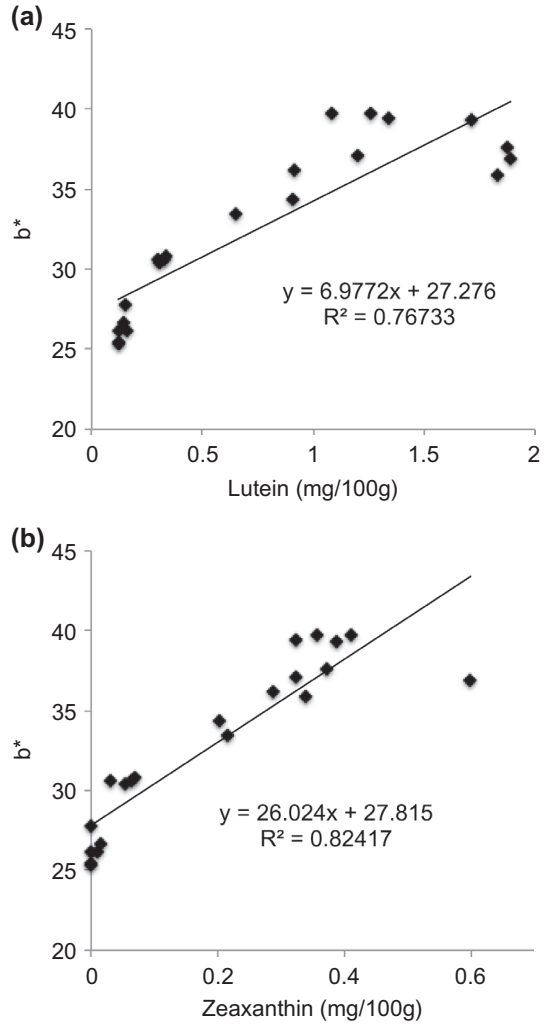

Figure 3. Correlation between yellow color and xanthophyll accumulation in foxtail millet grains. (a,b) The grains of foxtail millet cultivars and landraces listed in Table 1 were subjected to HPLC-PDA and the strength of the yellow color was quantified by colorimetry (b* value). The correlation between b*value and the amounts of lutein and zeaxanthin are indicated as scatter plots. Linear regression analysis was performed; the correlation curve and R^2 are indicated in the graphs.

of 1000 °C, the seed weight was saturated and the water contents decreased to under 30% (Figure S2).

Discussion

Lutein and zeaxanthin are natural yellow pigments in plants and green algae. These compounds are structural components of Photosystem II, and function as free radical scavengers to protect plants from photo-oxidative damage (Jahns & Holzwarth, 2012; Niyogi et al., 1997). Lutein is the most abundant xanthophyll in photosynthetic tissue (Jahns & Holzwarth, 2012). Green vegetables such as spinach are the major dietary source of lutein for humans (Sommerburg et al., 1998). Zeaxanthin is a functional component of the xanthophyll cycle and is converted to violaxanthin and/or carotene (Jahns & Holzwarth, 2012) depending on the light intensity. These pigments reduce photodamage of photosynthetic tissues.

It is not completely clear why xanthophylls accumulate in grains. Xanthophyll contents vary among foxtail millet accessions, and some white-grain foxtail millets accumulate almost no xanthophyll (Table 1). In foxtail millet cultivars and landraces that accumulate xanthophylls, lutein and zeaxanthin are localized in the grain endosperm. We expected that xanthophylls would accumulate in the bran to protect embryo from oxidation, but the data clearly indicate that they accumulate in the endosperm (Figure 3).

Xanthophylls are important for human health. Lutein is the most abundant carotenoid in human serum (Khachik et al., 1997a). Lutein is believed to have broad anti-inflammatory effects, and can reduce the risk of developing cardiovascular diseases (Ciccone et al., 2013). Zeaxanthin is present in serum and tissues and may have beneficial roles in cancer prevention and cardiovascular health (Khachik et al., 1997b; Kowluru et al., 2008; Widomska & Subczynski, 2003). Lutein and zeaxanthin are the major eye pigments in the macular lutea (Ciccone et al., 2013). These xanthophylls protect photoreceptors from oxidative damage (Khachik et al., 1997a; Kowluru et al., 2008; Sommerburg et al., 1998; Widomska & Subczynski, 2003). Xanthophyll intake from the diet or nutritional supplements increases macular pigment concentrations (Bone et al., 2003). Many intervention studies have shown that xanthophyll ingestion reduces the risk of eye diseases, including age-related macular degeneration (The AREDS2 Research Group, 2013a, 2013b, 2014).

Xanthophyll accumulation in unpolished grain (endosperm) is convenient from a nutritional viewpoint. The consumption of millet is recognized as beneficial for human health (Das et al., 2012; Dixit et al., 2011; Rao et al., 2011). Yellow foxtail millet varieties are thought to be more healthful than white millets due to their lutein and zeaxanthin contents. The consumption of 100-g yellow grains, e.g. those of Toranoo, Nisatai-zairai, Shinanotsubuhime, and Yuikogane, results in an intake of over 1.5-mg xanthophylls, in addition to xanthophylls from the general daily diet. An intake of 10-mg lutein and 2-mg zeaxanthin per day is recommended for healthy vision by AREDS2 (The AREDS2 Research Group, 2013a, 2013b, 2014); thus, 2-mg xanthophylls from foxtail millet would help in reaching this goal.

Our results provide important information for suppliers focused on cultivating xanthophyll-rich foxtail millet. Xanthophyll levels in foxtail millet grains change during the ripening stage, and colorimetry can be used to help decide when to harvest this crop (Figure 3). 'Yuikogane' accumulated the highest level of xanthophylls at 850 °C of accumulated temperature, although the grain was

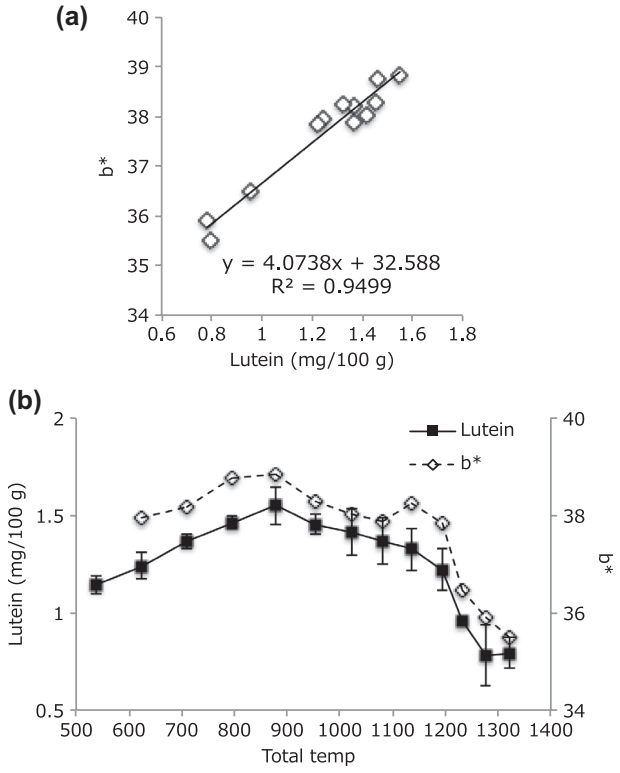

Figure 4. Yellow color and lutein accumulation in 'Yuikogane' grains during ripening. (a) Correlation between b* value and lutein contents in unpolished grains. Linear regression curve and R^2 are indicated in the graph. (b) Lutein contents and b* values during ripening plotted against accumulated temperature from heading.

still becoming heavily at this time (Figure S2). It should be harvested after 1000 °C of accumulated temperature when panicle weight and seed water content is taken into consideration. In order to obtain yellow grains, 'Yuikogane' should be harvested before 1150 °C (Figure 4b), because their xanthophylls content rapidly decreases if accumulated temperature exceeds 1200 °C. The decrease in xanthophylls content is considered to be due to excessive exposure to light after ripening. In summary, 'Yuikogane' should be harvested when the accumulated temperature from heading ranges from 1000 to 1150 °C and the b* value is approximately 38 or more.

Genetic factors are also a major consideration in xanthophyll accumulation, and breeding yellow millet is important for obtaining healthier grains. The complete genome foxtail millet has been sequenced (Zhang et al., 2012). Millet crops are salt-, cold-, and drought-tolerant, and they grow well in arid and semi-arid areas (Barton et al., 2009; Lu et al., 2009). Millet is a sustainable agricultural crop that can adapt to global climate change owing to its high stress tolerance (Hannah et al., 2013). The availability of the foxtail millet genome sequence will enable future breeding and biotechnology programs to generate foxtail millet cultivars and lines containing higher levels of xanthophylls, which would be highly desirable for future agricultural applications (Bennetzen et al., 2012; Zhang et al., 2012).

Conclusions

Polished grains of foxtail millet (*S. italica* (L.) P. Beauv.) contain xanthophylls, lutein, and zeaxanthin. Some cultivars and landraces contain over 2 mg per 100 g of xanthophylls. Xanthophyll levels vary depending on the cultivar, landrace, and time of harvest. Xanthophyll levels in foxtail millet grains can be estimated by measuring the yellow color of grain color using a colorimeter.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported in part by Grants-in-Aid for Scientific Research [grant number 26670910] from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Special Scheme to Deploy Highly Advanced Technology for Agriculture, Forestry and Fisheries (Proof study for the functional indication of lutein such as the spinach).

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