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研究課題名（和文）Application of body time detection by molecular timetable method for quality control of postharvest fruit and vegetables

研究課題名（英文）Application of body time detection by molecular timetable method for quality control of postharvest fruit and vegetables

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研究成果の概要（和文）：大豆モヤシの分子時刻表の構築が成功し，時計遺伝子・品質変化関連遺伝子の発現に及ぼす温度・CA貯蔵の影響について検討した。qPCR法で得られた遺伝子発現量をコサイン曲線式でフィッティングし，暗所貯蔵条件下における大豆モヤシにおいて概日リズムが存在することを見出した。20 ℃貯蔵では，時計遺伝子発現に規則的な位相と振幅を持つ有意なリズムが観察された。一方，10 ℃貯蔵は，各遺伝子の位相と振幅に深刻な影響を与え，概日時計機能が乱された。また，低O2ならびに高CO2貯蔵では，呼吸速度が減少し，重量減少や褐変の発生などの収穫後品質を維持したが，概日リズムは確認できたものの，大気貯蔵とは位相が異なった。

研究成果の学術的意義や社会的意義
The link between clock disruption and quality maintenance of soybean sprouts was first reported. The knowledge about the existence of the circadian clock and its response behavior during storage will help to develop a new postharvest preservation technology by controlling circadian rhythm.

研究成果の概要（英文）：The molecular timetable of soybean sprouts was proposed. The effect of temperature and CA storage on the clock genes and quality attributes and their related genes were examined. By fitting the gene expression level with the cosine curve equation, it was successfully found that the circadian rhythm existed under constant dark storage conditions of soybean sprouts. A significant rhythm in clock gene expression was observed in control. In contrast, low temperature storage diminished the cyclic expression of clock gene expression. Additionally, high CO2 concentrations during storage disturbed the circadian clock by affecting the phase and amplitude of each gene; for low O2, it was only affected by amplitude. Interestingly, low temperature, low O2, and high CO2 maintained postharvest quality, including reduced respiration, weight loss and browning incidence. The expression behaviors of postharvest quality attribute-related genes were also influenced by the storage treatments.

研究分野：Postharvest Physiology

キーワード：Circadian rthytm　Fresh produce　Quality control

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１．Research Background （研究開始当初の背景）

Circadian clock (CC) system produces a circadian rhythm (CR) by the expression of CC genes, which coordinating the internal time with the external environment in 24-h cycle a day. We previously found that the extension of shelf life under low temperature storage likely has a close relation to the CR statein soybean sprouts. However, the mechanism is not clear and the detection tool of CR in fresh produce is not available.

２．Research Purpose （研究の目的）

This study aims to propose the method for detection of CR state in fresh produce from CC gene expression and to determine the effect of temperature and CA (low O_2 and high CO_2) stresses on CC gene expression as well as the quality changes during postharvest storage. Here, soybean sprouts were selected, one of the most popular vegetables in Asian countries.

３．Research Methods（研究の方法）

３．１ Plant materials (soybean sprout cultivation)（材料および栽培条件）

Soybean seeds (*Glycine max*, cultivar “BS5012”) were used for cultivating sprouts. The cultivation practices were carried out in darkness. The cultivation was conducted in an incubator set at 25 °C for 4 days (approx. 96 h), where time counting was started from the seed soaking procedure, to produce a hypocotyl length of 8 ± 2 cm. Four-day-old sprouts were then harvested and used for gene expression and quality assessment.

３．２ Peak time and molecular timetable construction（PT と MTM の構築）

The expression levels of 47 target genes were measured by qPCR method. The time-course expression level of target clock genes was fitted to the cosine curve with a 24-h cycle. The 37 genes which expressed periodically were selected and these genes were assumed to be expressed under normal CR. The peak of the cosine curve was defined as peak time (PT). Molecular timetable (MTM) was obtained by plotting the PT and the normalized expression level of each selected gene. This plotting was then applied to the cosine curve, and the peak of the curve at this time was defined as body time (BT).

３．３ Storage condition（貯蔵条件）

A total of approx. 1700 harvested sprouts were divided into four groups (425 sprouts each) and placed in chambers (10 mm thick acrylic cylinders, 4.8 L) that were used for the following storage treatments: control (20 °C-air), low O_2 (20 °C-5% O_2), high CO_2 (20 °C-15% CO_2 + 20% O_2), and low temperature (10 °C-air) with 80 ± 5% relative humidity. All the sprouts were stored under constant dark conditions because light can have adverse effects on soybean sprout quality. The experiments were conducted for 24 h in all treatments. The gas compositions of all treatments were controlled using a flow-through system through a mass flow controller, and the percentage of O_2 and CO_2 was monitored by O_2 and CO_2 sensors. The gas flow rate through the chamber was 100 mL min^{-1}.

３．４ Measurements of quality attributes, clock genes, and quality related genes during storage（品質特性品質特および品質変化関連遺伝子・時計遺伝子の発現解析）

Respiration rate, weight loss, browning occurrence were measured at 0 h and 24 h of storage. Expression levels of clock genes and quality related genes were conducted at 4 h interval for 24 h. The reference gene (membrane-binding domain (Fab1/YOTB/Vac1/EEA1)-encoding genes, *FYVE*), and circadian clock genes (LHY-CCA1-LIKE 1-encoding gene, *LCL1*; TIMING OF CAB EXPRESSION 1-encoding gene, *TOC1*; GIGANTEA-encoding gene, *GI*; LUX ARRHYTHMO-encoding genes, *LUX*; and PSEUDO-RESPONSE REGULATOR-encoding gene, *PRR7*) were used in this study. Clock output genes related to postharvest physiology including fumarate hydratase 1 (*FUM1*), citrate synthase (*CS*), and 2-oxoglutarate dehydrogenase (*2-OGDH*), polyphenol oxidase 1 (*PPO1*), phenylalanine ammonia-lyase (*PAL*), and dehydration-responsive element-binding 5 (*DREB5*) were also used.

４．Results and Discussion（研究成果）

４．１ Peak time and molecular timetable

The coefficient of determination of fitting the experimented BT dataset (n=1) with the predicted BT (n=4) showed a value of 0.93, and the RMSE was 2.0 (h), so that the BT could be sufficiently estimated. Overall, it revealed that there is probability to construct a MTM by expression data of 37 time-indicating genes. Construction of MTM can be

possibly conducted without relying on comprehensive gene analysis by RNA sequencing.

４．２ Respiration rate（呼吸速度）

Figure 1 (A) represents the CO_2 production rate of soybean sprouts stored at control, low temperature, low O_2, and high CO_2 in constant dark. After 24 h of storage, the CO_2 production rate of soybean sprouts stored under low temperature, low O_2, and high CO_2 was significantly lower than that of the control. In this study, all treatments suppressed the respiration rate, which subsequently alleviated the quality deterioration of soybean sprouts compared to ambient air storage.

４．３ Weight loss（重量損失）

The effect of various storage treatments on the weight loss of soybean sprouts is presented in Figure 1 (B). After 24 h of storage, 4.08% weight loss was found in the control samples, whereas lower levels of weight loss at 2.08%, 2.82% and 2.54% were recorded under low temperature, low O_2 and high CO_2 storage conditions, respectively. This is likely the result of a lower respiration rate of soybean sprouts observed in the low temperature and CA treatments compared to the control soybean sprouts.

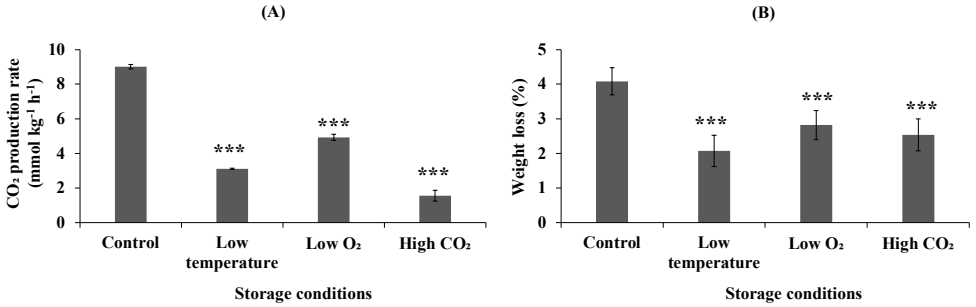

Figure 1 Effect of low temperature, low O_2, and high CO_2 on (A) CO_2 production rate (n = 3), (B) weight loss (n = 10) of soybean sprouts after 24 h storage in constant dark conditions. Bars for each group with an asterisk indicate statistically significant differences using the Dunnett test (*P <0.05, **P <0.01, ***P <0.001). Data presented are the mean ± SD.

４．４ Browning index（褐変変化）

Although slight browning symptoms were observed in soybean sprout cotyledons under ambient air temperature storage (control) after 24 h, a lower browning index (subjectively assessed) was detected in low temperature, low O_2 and high CO_2 treated sprouts than in the control (Fig. 2A). Similarly, the browning index derived from the L*, a*, and b* values also showed significant decreases in all treatments, which was concomitant with less browning (Fig. 2B). Since the results obtained from both measurement methods were highly correlated ($R^2 = 0.87$) (Fig. 2C), evaluation of soybean sprout browning by the visual scoring method could be used along with objective assessment.

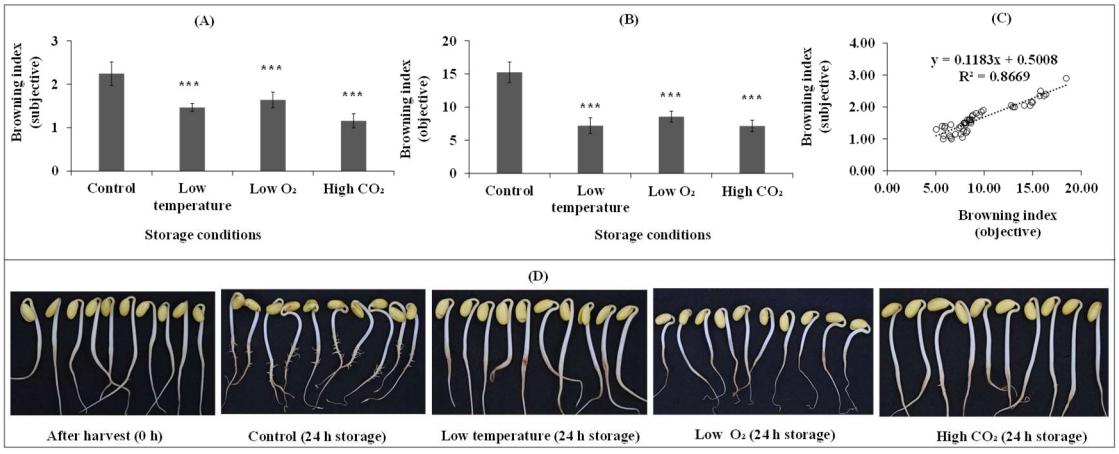

Figure 2 Effect of low temperature, low O_2, and high CO_2 on (A) browning index (subjective) (n = 12) (after 24 h storage), (B) browning index (objective) (n = 5) (after 24 h storage), and (C) relationship between subjective and objective data of browning index, and (D) appearance of soybean sprouts in constant dark conditions. Bars for each group with an asterisk indicate statistically significant differences using the Dunnett's test (*P <0.05, **P <0.01, ***P <0.001).

４．５ Evaluation of rhythmic pattern of clock gene expression（時計遺伝子の周期性）

To investigate the individual rhythmic behavior of soybean clock genes stored under ambient temperature (control), low temperature, low O_2, and high CO_2 in constant dark conditions, the measured values of each gene expression data were fit to a general cosine

curve. Regression cosine curves were determined by a cosinor analysis, and the R^2 values are shown in Figure 3. The significance of the rhythm was tested using P values obtained from the slope test. According to Figure 3 (A, C, D), the expression levels of clock genes (*GmLCL1, GmPRR7, GmGI,* and *GmLUX*) showed rhythmic waves with R^2 values of 0.30-0.70 ($P < 0.001$) when soybean sprouts were stored under control and CA (low O_2 and high CO_2) conditions. On the other hand, the significant wave form was unnoticeable for genes under low temperature conditions except *GmGI* and *GmLUX*. As a result, both genes (*GmGI*, *GmLUX)* maintained the corresponding behavior ($R^2 > 0.3$, $P < 0.001$), and other genes showed feeble rhythmicity with an R^2 value of ~0.20 ($P < 0.05$) at low temperatures (Fig. 3B). However, *GmTOC1* expression under low O_2 conditions displayed arrhythmicity similar to that of the control, with the lowest R^2 value of 0.04 ($P > 0.05$). Overall, this section describes how fitting of a cosine curve can be used to visualize circadian data in each condition that would be appropriate for rhythmicity assessment.

It is well documented that cosinor analysis is a classic approach for estimating the pattern of smooth rhythm even though the data points are relatively short (Cornelissen, 2014). The cosinor-based model assumes that the expression level of a gene is a sine or cosine function of the circadian time. Circadian profiles are considered rhythmic when a significant cosine fit (R^2) is confirmed. Recently, statistical support for the presence of significant circadian rhythmicity was evaluated by the cosinor-based method at a P value

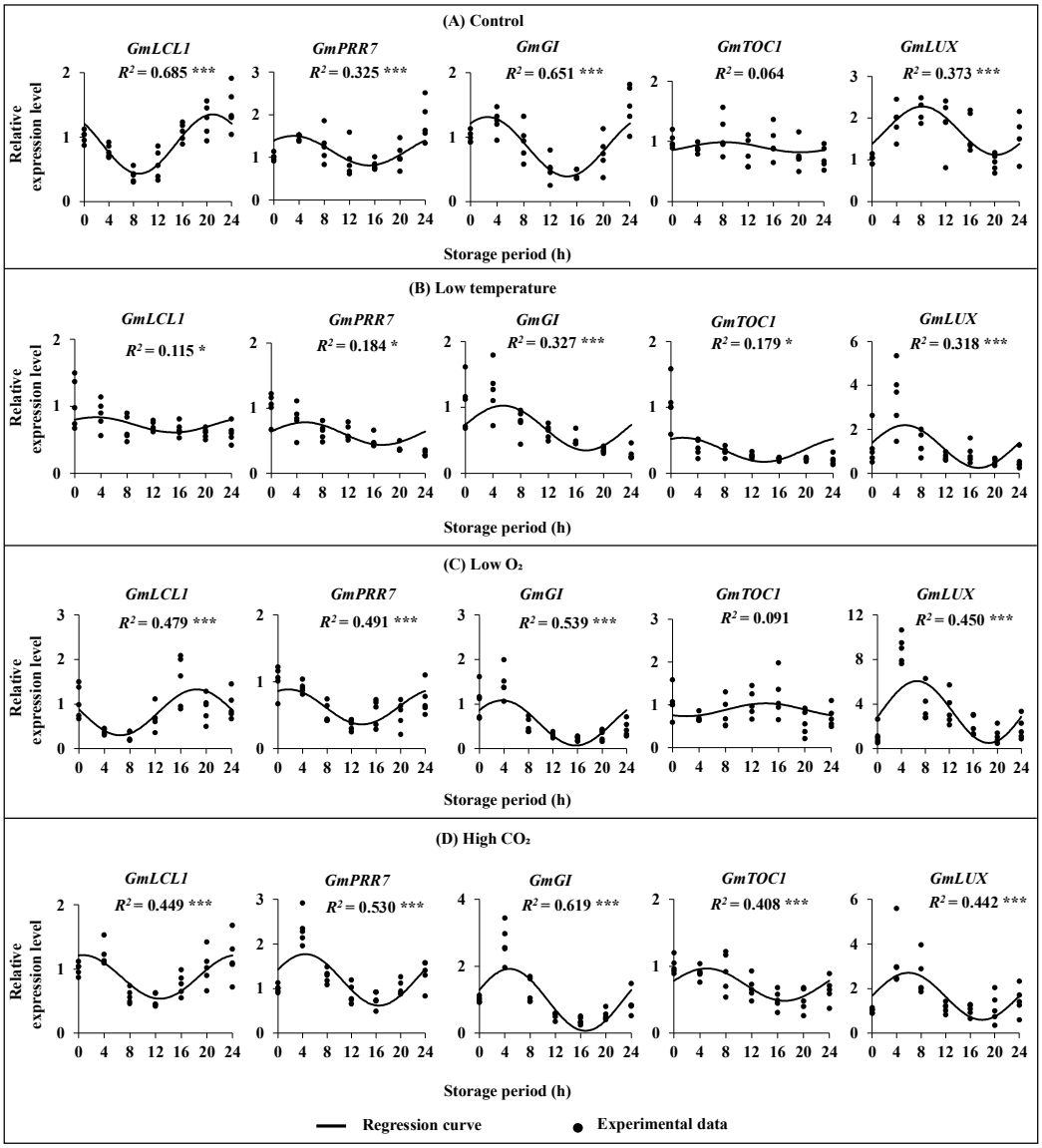

Figure 3 Fitting curve of relative clock gene expression with the cosine curve equation under different storage conditions: (A) control, (B) low temperature, (C) low O_2, and (D) high CO_2. The target genes of soybean sprout under different storage conditions were *GmLCL1*, *GmPRR7*, *GmGI, GmTOC1*, and *GmLUX*. R^2 stands for determination of coefficient and significancy of the R^2 values were tested using P value by slope test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The solid line indicates the regression cosine curve obtained from nonlinear regression. Various plots indicate relative gene expression values measured using qPCR from five biological replicates.

less than 0.01 (Ding et al., 2022; Parsons et al., 2020). In addition, P <0.001 was also applied for testing circadian rhythmicity in urinary bile acid of rats using cosinor analysis (Kawai et al., 2020). To confirm the presence of circadian rhythmicity in soybean sprouts during storage, P <0.01 was used as a statistical significance cutoff to declare circadian rhythmicity. To the best of our knowledge, this analysis has not yet been applied in postharvest storage conditions. This is the first report that statistically confirms the significant rhythm of clock gene expression in postharvest storage of soybean sprouts even under constant dark conditions.

４．６ Behaviors of postharvest physiological attributes-related gene expression（品質変化関連遺伝子の発現）

Circadian output pathways provide a link between the circadian oscillator and various physiological and biological processes of plants. The expression of target genes which relate to postharvest physiological attributes were analyzed to prove a link between circadian clock and changes in postharvest quality. Gene relating to respiratory pathway (TCA cycle) intermediates (*GmFUM1*, *GmCS*, *Gm2-OGDH*), browning producing enzymes (*GmPPO1*, *GmPAL*), and drought or cold stress responsive factor (*GmDREB5*) of soybean sprouts were analyzed as clock output gene expression at 4 h intervals for 24 h storage. Under control condition, the expression of *GmFUM1*, *GmCS*, *Gm2-OGDH*, *GmPPO1* and *GmPAL* showed a wave fluctuation with a broad peak shape at 8 h to 12 h of storage, which might indicate an active cellular function under ambient temperature storage (Fig. 4). In contrast, the expression levels of these genes were consistently suppressed by low temperature, low O_2, and high CO_2 storage treatments (Fig. 4). The expression level of *GmDREB5* under low temperature treatment was slightly higher than that of the control. Nevertheless, the peak of expression level of all storage treatments was not remarkably detected during 24 h storage (Fig. 4F). The results suggested that *DREB* gene of soybean sprouts might be either clock independent and/or not responsive during postharvest stress.

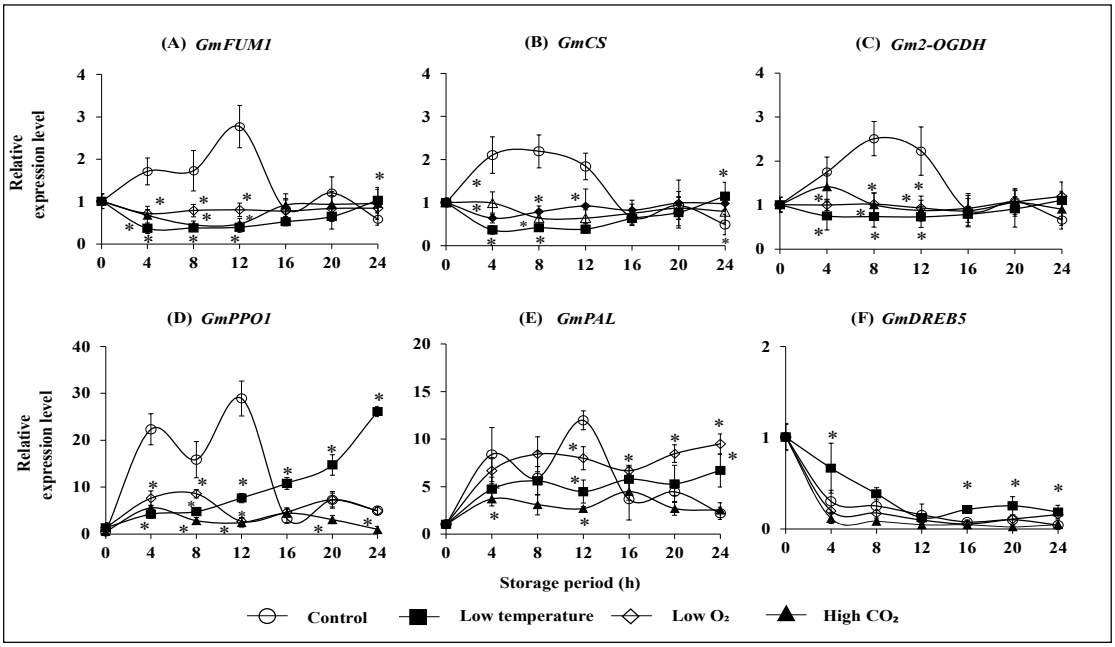

Figure 4 Effect of low temperature, low O_2, and high CO_2 on clock output genes related to different postharvest physiological attributes; (A) *GmFUM1*, (B) *GmCS*, (C) *Gm2-OGDH*, (D) *GmPPO1*, (E) *GmPAL*, and (F) *GmDREB5*. Data presented are the mean of five replicates ± SD. (*) indicates significant difference between control and treated samples in each time point (Dunnett's test, P <0.05).

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Ding, H., Meng, L., Xing, C., Esser, K.A., Huo, Z., 2022. Statistical methods for detecting circadian rhythmicity and differential circadian patterns with repeated measurement in transcriptomic applications. bioRxiv 2022.06.05.494875.

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５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

１．著者名 Shomodder, A., Imaizumi, T., Nagata, M., Kasai, E., Shiina, T., Tsuta, M., Thammawong, M.** and Nakano, K.*	４．巻 281
２．論文標題 Existence of circadian rhythm and its response behavior under different storage conditions of soybean sprouts	５．発行年 2023年
３．雑誌名 Journal of Plant Physiology	６．最初と最後の頁 153906
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.jplph.2022.153906	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計2件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 佐藤舞, THAMMAWONG Manasikan, 中野浩平
２．発表標題 大豆モヤシの収穫操作による時計遺伝子の再起にかんする検討
３．学会等名 2023 年農業施設学会 学生・若手研究発表会
４．発表年 2023年

１．発表者名 佐藤舞, THAMMAWONG Manasikan, 中野浩平
２．発表標題 大豆モヤシの収穫操作による時計遺伝子の再起にかんする検討
３．学会等名 2023_農業食料工学会 食料・食品工学部会シンポジウム
４．発表年 2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関