Parthenocarpy Induced Fluctuations in Pungency and Expression of Capsaicinoid Biosynthesis Genes in a Japanese Pungency-variable Sweet Chili Pepper ‘Shishito’ (Capsicum annuum)

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The pungency-variable sweet chili pepper ‘Shishito’ (Capsicum annuum) is widely cultivated in Japan. While ‘Shishito’ is generally used as a vegetable because of its relatively low pungency, it sometimes exhibits high pungency depending on cultivation conditions. Although the occurrence of pungent ‘Shishito’ fruits is a problem in retail distribution and utilization, the responsible mechanism is largely unknown. As one approach to clarify the mechanism, we focused on the effects of parthenocarpy (resulting in seedless fruit) on the pungency traits of ‘Shishito’ fruits. In the present study, artificial parthenocarpic ‘Shishito’ fruits were prepared by treatment with 2,4-dichlorophenoxyacetic acid (2,4-D), and the pungency level was investigated by quantification of capsaicinoids, components responsible for the pungency. For comparison, two controls were used: naturally pollinated fruits and 2,4-D-treated pollinated fruits which were exposed to 2,4-D treatment without parthenocarpy. The results indicated that the capsaicinoid content in parthenocarpic fruits tended to be higher compared to the controls. This suggests that the alterations in pungency in ‘Shishito’ were associated with parthenocarpy. Further, these relationships were assessed using a molecular method, and gene expression analysis using qRT-PCR was conducted on 16 capsaicinoid biosynthesis genes. The results showed that eight capsaicinoid biosynthesis genes (Pun1, pAMT, KAS, CaMYB31, BCAT, CuKR1, ACL, and FAT) exhibited parthenocarpic-specific high expression, suggesting that these genes influence capsaicinoid biosynthesis and the pungency levels in parthenocarpic fruits. This is a novel report that carefully investigated the parthenocarpic-dependent changes in the pungency traits of chili pepper. We anticipate that the data will add to horticultural knowledge and help control the pungency of ‘Shishito’ fruits during cultivation.

Key Words: capsaicinoid biosynthesis genes, capsaicinoids, 2,4-dichlorophenoxyacetic acid, gene expression analysis, seeds.

Introduction

Chili peppers, members of the Solanaceae family, are globally cultivated and consumed as vegetables or spices. Among the Solanaceae, chili pepper is characterized by its hot taste (pungent flavor), and chemical analogs known as capsaicinoids contribute to these pungency traits (Suzuki et al., 1980). Capsaicinoids mostly accumulate in the epidermal cells of the placental septum in fruits (Iwai et al., 1979), and the contents are proportional to the pungency level. Biosynthesis of capsaicinoids is accomplished by condensation of vanillylamine and branched-chain fatty acid moieties, and these two precursors are derived from two separate pathways that constitute the capsaicinoid biosynthesis pathway (Arce-Rodriguez and Ochoa-Alejo, 2019). One is the phenylpropanoid pathway, which synthesizes vanillylamine from phenylalanine, and the other is the
branched-chain fatty acid pathway, which synthesizes a series of branched-chain fatty acid moieties from valine or leucine. Additionally, several genes are known to participate in the capsaicinoid biosynthesis pathway (Qin et al., 2014). Above all, Pun1, which encodes an acyltransferase, is one of the most critical genes in capsaicinoid biosynthesis and is responsible for the final reaction in the pathway (Stewart et al., 2005, 2007). Then, putative aminotransferase (pAMT), which produces vanillylamine from vanillin, is also important and mutation of this gene caused a drastic reduction in capsaicinoids (Koeda et al., 2014; Lang et al., 2009; Tanaka et al., 2010, 2019). Furthermore, in recent years two novel genes were discovered that induced loss of pungency. One is CaMYB31, encoding an R2R3-MYB transcriptional factor, which was proposed to directly regulate the expression of several capsaicinoid biosynthesis genes (Arce-Rodríguez and Ochoa-Alejo, 2017; Zhu et al., 2019). The other is ketoacyl-ACP reductase (CaKRI), which participates in the branched-chain fatty acid pathway (Koeda et al., 2019). In addition to these four critical genes, other genes in the pathway are also involved in capsaicinoid biosynthesis, and their genotypes or gene expressions are considered to affect on the amount of capsaicinoids in fruits (Zhang et al., 2016; Han et al., 2018).

According to previous research, the amount of capsaicinoids in chili pepper fruits is determined not only by the variety’s genotype, but also by fruit development and cultivation conditions (Arce-Rodríguez and Ochoa-Alejo, 2019). Previous studies reported some cultivation conditions resulted in changes in pungency traits. Firstly, the effects of water stress have been well investigated. Briefly, Haak et al. (2012), Phimchan et al. (2012) and Sung et al. (2005) reported that water deficiency increased capsaicinoid contents, and Phimchan et al. (2014) observed that some capsaicinoid biosynthesis enzymes, such as phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and capsaicinoid synthase (CS), exhibited higher activities under drought stress. Further, temperature and light exposure are also reported to affect pungency traits. High temperatures were reported to cause fluctuations in pungency, although the level of fluctuation differed according to the chili pepper cultivar (González-Zamora et al., 2013). Meanwhile, light exposure using blue LEDs increased the capsaicinoid contents in C. annuum ‘Cheonnyang’ (Gangadhhar et al., 2012). In addition to the above effects, nitrogen and potassium fertilization and the atmospheric CO₂ concentration were also reported to cause changes in the pungency level of fruits (Garruña-Hernández et al., 2013; Medina-Lara et al., 2008). Therefore, the amount of capsaicinoid contents in chili pepper changes depending on various environmental conditions during cultivation.

As for the Japanese pungency-variable sweet chili pepper ‘Shishito’ (C. annuum), its pungency traits exhibited unique responses to environmental effects. In Japan, ‘Shishito’ is widely cultivated and utilized as a vegetable due to its relatively low pungency. However, some of the peppers have high pungency, creating issues in retail distribution and utilization. The high pungency is caused by changes in cultivation conditions, and Murakami et al. (2006) reported consecutive light conditions and high temperature under a dark condition increased the ratio of pungent fruits. It is known empirically that ‘Shishito’ with seedless fruits tends to be pungent. Specifically, parthenocarpic (lacking seeds) fruits are called ‘stone fruits’, which describes their small seedless fruit, and they frequently have high pungency. According to Ishikawa et al. (2004), seedless ‘Shishito’ fruits were reported to contain higher capsaicinoid contents than normally pollinated (seeded) fruits. Doi et al. (2011) conducted multivariate analysis of relations between environmental factors and pungent fruiting of ‘Shishito’, and suggested a decrease in seeds was one of the factors that contributed to occurrence of pungent ‘Shishito’ fruits. Therefore, parthenocarpic is thought to be related to the pungency traits of ‘Shishito’. However, besides these reports, little detailed information is available regarding parthenocarpic-dependent pungency changes in ‘Shishito’ and the responsible mechanism is largely unknown. The present study thus aimed to carefully investigate the relationship between pungency traits and parthenocarpy in chili peppers, by examining the capsaicinoid contents and expression of capsai‐
icoid biosynthesis genes in artificial parthenocarpic ‘Shishito’ fruits.

Materials and Methods

Plant materials and parthenocarpy treatment

‘Shishito’ (Takii & Co., Ltd., Kyoto, Japan) and a Japanese pungent variety ‘Takanotsume’ (Sakata seed Corporation, Yokohama, Japan) were prepared in this study. We sowed seeds of each variety in plastic trays with 200 holes on March 24, 2019, and the seedlings were firstly transplanted to plastic pots 72 mm diameter, and then transplanted to clay pots of 30 cm diameter. The plants were cultivated at a greenhouse in Shinsu University (Nagano, Japan) throughout. The temperature was maintained 15–33°C and light intensity was reduced to 40% of full sunshine by using a shading seat. In the cultivation, we used commercially available culture soil (Naeichiban; Sumitomo forestry landscaping Co., Ltd., Tokyo, Japan). The plants were manually watered three or five times a day, and the soil surface was kept humid. For fertilization, 10 g of mixed granule fertilizer (Kumiai granular composite N552s [N:P₂O₅:K₂O = 15:15:12]; JA Agrivel Nagano, Nagano, Japan) was applied every two weeks after first flowering. Before parthenocarpy treatment as mentioned below, flower buds were constantly removed to facilitate growth of the plants.

Parthenocarpy treatment of ‘Shishito’ was performed
from September 5 to 10, 2019. In parthenocarpy treatment, 2,4-dichlorophenoxyacetic acid (2,4-D) (11831-22; Nacalai tesque Inc., Kyoto, Japan) was used as the hormone for fruiting. Firstly, the petals, stamen and stigma were removed from the flower bud a day before flowering to prevent pollination. Then, a cotton bud soaked in 2,4-D solution (20 ppm) was placed on the ovary of the flower bud. We confirmed that the ovary was completely covered with 2,4-D solution. Thereafter, parthenocarpic fruits were harvested 20, 35, and 50 days after flowering (DAF). As controls, naturally pollinated fruits and 2,4-D-treated pollinated fruits were prepared. Naturally pollinated fruits were obtained by normal pollination. For the 2,4-D-treated pollinated fruits, the 2,4-D solution was placed on the ovary a day after flowering; this treatment resulted in the development of pollinated (seeded) fruits that had undergone 2,4-D treatment. Additionally, we also prepared naturally pollinated fruits of ‘Takanotsume’ to reference the pungent traits and gene expression of a pungent variety. These controls were harvested at the same fruit development stages as the parthenocarpic fruits.

**Morphological survey of fruits**

In the parthenocarpic fruits, naturally pollinated fruits and 2,4-D-treated pollinated fruits of ‘Shishito’, the morphological traits were investigated at 20, 35, and 50 DAF. In the survey, the number of seeds, fruit length, fruit width, and fresh weight of whole fruits were measured. In addition, the placental septum was separated from the fruit and the fresh weight was measured.

**Capsaicinoid extraction and HPLC analysis**

Following the morphological survey of fruits, the placental septum was used for extraction of capsaicinoids. Firstly, the placental septum was lyophilized using a freeze-dryer (FDU-200; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). After determining the dry weight of samples, they were placed (0.09 to 0.25 g) into collection tubes with stainless-steel bases, and crushed using a Micro Smash™ MS-100 (TOMY SEIKO Co., Ltd., Tokyo, Japan). Then, capsaicinoids (capsaicin and dihydrocapsaicin) were extracted in 5 mL acetone. After transferring the supernatant to an eggplant-shaped flask, 2 mL ethyl acetic was added to the residue and the supernatant was collected. The mixed supernatant was set to 1.0 mL·min⁻¹ and 40°C, respectively. As for detection with a UV detector, the wavelength was set at 280 nm. The capsaicinoid concentration (μg·g⁻¹DW) was calculated based on the peak area of capsaicin and dihydrocapsaicin. Additionally, we also investigated capsaicinoid contents for one tissue sample of the placental septum (μg/fruits) and the contents were calculated by multiplying the capsaicinoid concentration (μg·g⁻¹DW) and dry weight of each placental septum (gDW).

**RNA isolation and qRT-PCR of capsaicinoid biosynthesis genes**

Using an RNAeasy Plant Mini Kit (Qiagen, Hilden, Germany), total RNA was isolated from the placental septum of parthenocarpic fruits, 2,4-D-treated pollinated fruits of ‘Shishito’ and naturally pollinated fruits of ‘Shishito’ and ‘Takanotsume’, according to the manufacturer’s instructions. Next, total RNA was used for reverse transcription PCR, and cDNA was synthesized using a High-Capacity RNA-to-cDNA kit (Applied Biosystems, MA, USA).

qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems). In the analysis, 16 capsaicinoid biosynthesis genes were analyzed as target genes, and actin was used as the reference gene. For gene amplification, we used the primers described by Tanaka et al. (2017), Koeda et al. (2019) and Han et al. (2019), and complete sequences are listed in Table 1. In the PCR, PowerUp™ SYBR Green Master Mix (Applied Biosystems) was used as the PCR mixture, and the thermal cycle followed that described by Tanaka et al. (2017) (98°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 68°C for 60 s). Finally, relative expression was calculated based on the comparative CT method.

**Results**

**Morphological traits of parthenocarpic fruits**

Naturally pollinated fruits and 2,4-D-treated pollinated fruits had elongated fruits containing multiple seeds, whereas parthenocarpic fruits had a stumpy shape and no seeds (Table 2; Fig. 1). The fruit length of parthenocarpic fruits was shorter than that of the other two groups throughout fruit development (Fig. 2A), although fruit width did not differ significantly among all treatments (Fig. 2B). Regarding temporal changes, the parthenocarpic fruits showed almost no changes in either fruit length or width, while both the naturally pollinated fruits and the 2,4-D-treated pollinated fruits showed gradual increases from 20 to 50 days after flowering (DAF). Similar results were also observed for the fresh weight of the placental septum and whole fruits. Naturally pollinated fruits and 2,4-D-treated fruits had elongated fruits containing multiple seeds, whereas parthenocarpic fruits had a stumpy shape and no seeds (Table 2; Fig. 1). The fruit length of parthenocarpic fruits was shorter than that of the other two groups throughout fruit development (Fig. 2A), although fruit width did not differ significantly among all treatments (Fig. 2B). Regarding temporal changes, the parthenocarpic fruits showed almost no changes in either fruit length or width, while both the naturally pollinated fruits and the 2,4-D-treated pollinated fruits showed gradual increases from 20 to 50 days after flowering (DAF). Similar results were also observed for the fresh weight of the placental septum and whole fruits. Naturally pollinated fruits and 2,4-D-treated
pollinated fruits showed increases in fresh weight of whole fruits and placental septum from 20 to 50 DAF (Fig. 2C, D, respectively). However, the weights of parthenocarpic fruits showed almost no changes after 20 DAF.

**Capsaicinoid contents in ‘Shishito’ parthenocarpic fruits**

We compared the capsaicinoid concentration in the placental septum among the three ‘Shishito’ treatments. In parthenocarpic fruits, capsaicinoid concentrations were 985 to 2,989 μg·g⁻¹DW, 548 to 3,204 μg·g⁻¹DW and 258 to 1,452 μg·g⁻¹DW at 20, 35, and 50 DAF, respectively, and these values were higher than those of the other treatments (Fig. 3A). In contrast, for the naturally pollinated fruits and 2,4-D-treated pollinated fruits, the capsaicinoid concentrations were less than 1,360 μg·g⁻¹DW at each time point. When we focused on the concentration range at each developmental stage, the parthenocarpic fruits consistently had the largest range among the three treatments. This indicated that the capsaicinoid concentration was largely different according to individual parthenocarpic fruits, and that these fluctuations are independent from those observed...
for the other treatments. On the other hand, we also compared the capsaicinoid content per one tissue sample of the placental septum, and the results were similar to those observed for capsaicinoid concentration, i.e., the capsaicinoid content of parthenocarpic fruits tended to be higher and showed large fluctuations compared to the other treatments (Fig. 3B). These results indicated that parthenocarpic fruits were more likely to be pungent than the other treatments, independent of the size of the placental septum or fruits.

Expression of capsaicinoid biosynthesis genes

qRT-PCR analysis of a total of 16 capsaicinoid biosynthesis genes (Fig. 4) was conducted using the placental septum in the three ‘Shishito’ fruit treatments. We additionally investigated and referenced the gene expression level in the Japanese pungent variety ‘Takanotosume’ to comprehensively investigate expression changes of the 16 genes in ‘Shishito’ fruits. The capsaicinoid concentrations in naturally pollinated fruits of ‘Takanotosume’ were 10,279 μg·g⁻¹DW, 23,015 μg·g⁻¹DW and 15,097 μg·g⁻¹DW at 20, 35, and 50 DAF, respectively (Fig. 5A) and these were always higher than those of ‘Shishito’ fruits in all treatments.

As a result, we divided the 16 genes into three groups (groups 1, 2, and 3) based on their expression patterns in the three ‘Shishito’ fruit treatments (Fig. 5B). *Pun1*, *pAMT*, *ketoacyl-ACP synthase (KAS)*, and *CaMYB31* were assigned to group 1, as these genes were highly expressed only in parthenocarpic fruits. Remarkably, parthenocarpic fruits at 20 DAF had the highest expression among all treatments and all time points. In contrast, gene expression remained relatively low in naturally pollinated fruits and 2,4-D-treated pollinated fruits at all time points. The genes *branched-chain amino acid transferase (BCAT)*, *CaKR1*, *acyl carrier protein (ACL)* and *acyl-ACP thioesterase (FAT)* were assigned to group 2. In common with group 1, group 2 genes exhibited slightly higher expression in parthenocarpic fruits, at least, expression levels at 20 DAF were consistently higher in parthenocarpic fruits compared to the other treatments. However, from 35 to 50 DAF, expression patterns of group 2 genes were different according to genes and treatments. For example, the expression levels of *BCAT* and *FAT* commonly in-
increased up to 50 DAF in all treatments, while those of CaKRI and ACL increased up to 35 DAF, but decreased at 50 DAF in naturally pollinated fruits and 2,4-D-treated pollinated fruits, although parthenocarpic fruits showed increases from 35 to 50 DAF. Finally, the other genes including acyl-CoA synthetase (ACS), branched-chain α-ketoacid dehydrogenase (BCKDH), 4-coumaroyl-CoA ligase (4CL), PAL, coumaroyl shikimate/quinate 3-hydroxylase (C3H), C4H, hydroxycinnamoyl transferase (HCT) and caffeic acid O-methyltransferase (COMT) were assigned to group 3. In contrast to group 1 and 2, group 3 genes did not exhibit parthenocarpic fruit-specific gene expression, and the expression levels did not differ greatly among any treatments. Additionally, gene expression tended to increase in a time-dependent manner. The expression levels of ACS, BCKDH, and 4CL increased from 20 to 50 DAF, while those of PAL, C3H, C4H, HCT, and COMT showed almost no changes between 20 to 35 DAF, but increased from 35 to 50 DAF.

When we focused on the differences in the expression levels between ‘Shishito’ and ‘Takanotsume’, naturally pollinated fruits of ‘Takanotsume’ at 20 DAF had higher expressions of group 1 and 2 genes compared to ‘Shishito’ fruits in all treatments. Therefore, it was obvious that naturally pollinated fruits and parthenocarpic ‘Shishito’ fruits had lower expressions of these genes, which was within a range not exceeding the expression levels of ‘Takanotsume’. However, group 3 genes were not always highly expressed in ‘Takanotsume’. For example, expression levels of ACS, BCKDH and HCT were almost the same for ‘Takanotsume’ and ‘Shishito’ throughout fruit development. However, several genes such as C3H and COMT were highly expressed in ‘Takanotsume’ at 20 DAF.

**Discussion**

In the present study, we focused on the parthenoecarpic-dependent fluctuations in pungency in the pungent-variable sweet chili pepper ‘Shishito’. Prior to our study, it was reported that ‘Shishito’ fruits had fluctuations in their pungency levels, and a lack of, or reduction in, seeds were suggested to be involved in these differences (Doi et al., 2011; Ishikawa et al., 2004). As one approach to investigate this phenomenon, we grew artificial parthenocarpic ‘Shishito’ fruits and examined their pungency traits. Firstly, we obtained artificial parthenoecarpic fruits grown by treatment with 2,4-D, and their capsaicinoid concentrations were compared with naturally pollinated fruits. We also prepared 2,4-D-treated pollinated fruits as a second control, because parthenoecarpic as well as 2,4-D treatment could influence the pungency traits. The results showed that the capsaicinoid concentration of parthenoecarpic fruits was higher than that of the other treatments, although there were large fluctuations among individual parthenoecarpic fruits (Fig. 3A). These results were consistent with the results of Ishikawa et al. (2004), who showed natural (non-artificial) parthenoecarpic ‘Shishito’ fruits had higher capsaicinoid concentrations than seeded fruits. Our results thus demonstrated that parthenoecarpic contributed to the in-
increases in capsaicinoids and fluctuations in the pungency of ‘Shishito’ fruits. However, no clear differences were observed in capsaicinoid concentrations between 2,4-D-treated pollinated fruits and naturally pollinated fruits. This indicated that 2,4-D has no or minimal effects on pungency traits compared to parthenocarpy.

Arce-Rodríguez and Ochoa-Alejo (2017) reported that indole-3-acetic acid (IAA) promoted increases in capsaicinoid biosynthesis genes in placental tissues in vitro, and suggested that hormones, including auxin,
had effects on capsaicinoid biosynthesis. In this context, 2,4-D, which is synthetic auxin, may exert effects on capsaicinoid biosynthesis; however, the effect was obviously smaller than that of parthenocarpy in the present study. Furthermore, we also investigated the capsaicinoid contents in single tissue samples of the placental septum. This is because placental size can affect capsaicinoid concentrations, i.e., a small placental septum exhibits higher capsaicinoids concentrations than a large one, even though the total capsaicinoid contents are the same. Notably, the size of each placental septum varied in the present study; therefore, it was necessary to confirm the total capsaicinoid contents. The results were similar to capsaicinoid concentrations, i.e., the parthenocarpic fruits showed higher capsaicinoid contents along with fluctuations among individual parthenocarpic fruits compared to the other treatments (Fig. 3B). This demonstrated that parthenocarpy caused the changes in pungency traits of ‘Shishito’ fruits, regardless of placental size.

A molecular method was employed to further investigate the parthenocarpic-dependent changes in pungency traits. In the present study, qRT-PCR analysis was conducted on a total of 16 capsaicinoid biosynthesis genes, and expression patterns were compared among the parthenocarpic fruits and the two controls. As a result, we classified the 16 genes into groups 1, 2, and 3 based on similarities in expression patterns. In group 1, the gene expressions of Pun1, pAMT, KAS, and CaMYB31 were higher in parthenocarpic fruits. In contrast, the above gene expression in naturally pollinated fruits and 2,4-D-treated pollinated fruits remained relatively low over the course of the study, indicating that parthenocarpy accounted for the changes in expression. In previous studies, the group 1 genes were reported to be necessary for capsaicinoid biosynthesis. For example, Pun1 is the most downstream gene in the capsaicinoid biosynthesis pathway, and functional loss of Pun1 due to mutation prevents capsaicinoids synthesis (Stewart et al., 2005). As a result, functional loss of CaMYB31 and pAMT also caused the loss of, or drastic reduction in, pungency (Han et al., 2019; Tanaka et al., 2019). Moreover, it was reported that gene silencing of Pun1, pAMT, KAS, and CaMYB31 using virus-induced gene silencing (VIGS) caused dramatic decreases in capsaicinoid contents (Abraham-Juárez et al., 2008; Arce-Rodríguez and Ochoa-Alejo, 2015, 2017). In this context, it was proposed that the expressions of Pun1, pAMT, KAS, and CaMYB31 influenced capsaicinoid biosynthesis in ‘Shishito’, and parthenocarpy-associated changes in their expression resulted in fluctuations in fruit pungency. Additionally, focusing on 20 DAF, such parthenocarpic fruit-specific high expression was also observed for the group 2 genes BCAT, CaKR1, ACL and FAT. In common with group 1, expression of these genes was also suggested to influence capsaicinoid biosynthesis. For ACL and FAT, their expressions were reported to be positively correlated with pungency levels (Aluru et al., 2003). Also, Tanaka et al. (2017) reported BCAT gene expression was relatively higher in pungent varieties compared to non-pungent varieties, and Koeda et al. (2019) revealed that gene-silencing of CaKR1 by VIGS caused reductions in capsaicinoids. Integrating their results, the expressions of group 2 genes were also correlated with pungency levels, and it was understandable that their high expressions contributed to fluctuations in pungency in parthenocarpic ‘Shishito’ fruits. Regarding these group 1 and group 2 genes, Doi et al. (2013) also reported increases in expression of pAMT, BCAT, KAS, ACL, FAT, and Pun1 when naturally pollinated ‘Shishito’ fruits had increased pungency levels. Our results were consistent with their results, and not only artificial parthenocarpic fruits, but also normal ‘Shishito’ fruits were likely to have expression changes when they exhibited high pungency. Furthermore, as for the parthenocarpic fruit-specific high expression observed in group 1 and group 2 genes, transcriptional control by CaMYB31 was considered. Zhu et al. (2019) recently proposed that an MYB transcriptional factor encoded by CaMYB31 directly regulates expression levels of capsaicinoid biosynthesis genes. In their report, a dual-luciferase reporter assay and ChiP-qPCR analysis revealed CaMYB31 directly targeted a set of capsaicinoid biosynthesis genes to activate their expressions. In our study, group 1 and group 2 genes consisted of CaMYB31 and its target genes (Pun1, pAMT, KAS, BCAT, and FAT), and their co-expression fluctuation was specifically observed in parthenocarpic fruits of ‘Shishito’ at 20 DAF (Fig. 5B). Considering this commonality, it was suggested that expression levels of group 1 and group 2 genes in ‘Shishito’ changed under transcriptional regulation by CaMYB31, and this regulation system contributed to the parthenocarpic-dependent changes in pungency traits. Interestingly, group 1 and group 2 genes, except for CaMYB31, are located downstream in the capsaicinoid biosynthesis pathway or the branched-chain fatty acid biosynthesis pathway (Fig. 4). It is likely that these genes are activated by CaMYB31 during early fruit development when pungency traits change. On the other hand, the genes assigned to group 3 (ACS, BCKDH, PAL, 4CL, C3H, C4H, HCT, and COMT) did not exhibit parthenocarpic-specific expression as observed for group 1 or 2, and the expression patterns were almost identical between parthenocarpic fruits and the other treatments. This demonstrated that the expression of these genes was hardly influenced by parthenocarpy. The majority of group 3 genes are associated with the phenylpropanoid pathway (Fig. 4), and it has been reported they are normally expressed regardless of the pungency level of fruits (Arce-Rodríguez and Ochoa-Alejo, 2017; Han et al., 2019; Tanaka et al., 2017). The phenylpropanoid pathway is known to be a biosynthesis pathway of various secondary metabolites such as phenolic acid,
flavonoids and lignins (Liu et al., 2015). Therefore, it is thought that phenylpropanoid biosynthesis genes are normally expressed regardless of capsaicinoid biosynthesis. At least, the present study did not reveal any differences in the expression of these genes among the three treatments, even though the parthenocarpic fruits showed changes in pungency traits.

To further understand the parthenocarpy-dependent changes in pungency traits, we referenced capsaicinoid concentrations and expression levels of capsaicinoid biosynthesis genes in the Japanese pungent variety ‘Takanotsume’. As a result, naturally pollinated fruits of ‘Takanotsume’ were found to have high capsaicinoid concentrations over 10,000 μg·g⁻³·DW throughout fruit development (Fig. 5A). These were higher than not only naturally pollinated fruits of ‘Shishito’, but also parthenocarpic fruits. Therefore, it appeared that ‘Shishito’ parthenocarpic treatment caused changes in the pungency traits, within a range not exceeding that of ‘Takanotsume’. In relation to this, expression levels of group 1 (Pun1, pAMT, KAS, and CaMYB31) and group 2 genes (BCAT, CaAKR1, ACL, and FAT) in ‘Shishito’ never exceeded the maximum levels in ‘Takanotsume’; ‘Takanotsume’ at 20 DAF exhibited the highest expressions of these genes (Fig. 5B). This indicated parthenocarpic-dependent expression changes in group 1 and group 2 genes were also within a range not exceeding that of ‘Takanotsume’. When we consider that parthenocarpic fruits of ‘Shishito’ showed lower capsaicinoid concentrations than ‘Takanotsume’, these results are reasonable. Therefore, such lower expressions of group 1 and group 2 genes may be responsible for the relatively low pungency of ‘Shishito’, and the pungency levels depended on their expression. However, it was observed that CaMYB31, KAS, pAMT belonging to group 1 genes were expressed in parthenocarpic fruits at 50 DAF (Fig. 5B). According to previous research, group 1 genes are commonly known to highly express in immature fruits, but hardly express at all at mature stages (Zhu et al., 2019). Even though ‘Takanotsume’ and naturally pollinated fruits of ‘Shishito’ exhibited these expression patterns, only parthenocarpic fruits of ‘Shishito’ did not follow such patterns, except for Pun1. We could not clarify why some group 1 genes were expressed even in mature fruits. However, ‘Shishito’ parthenocarpic fruits basically exhibited group 1 gene-specific expression peaks at 20 DAF (Fig. 5B), so that these fruits hypothetically exhibit a slow decrease in their expressions, and these expressions disappeared after 50 DAF. Further research is needed to investigate their expressional changes and pungency traits in parthenocarpic fruits after 50 DAF.

In this study, we revealed that parthenocarpic induced fluctuations in the pungency traits of ‘Shishito’. Although we investigated this phenomenon by investigating artificially produced parthenocarpic fruits, our results clearly supported the previous research (Doi et al., 2011; Ishikawa et al., 2004), suggesting that a lack of, or a decrease in, seeds causes changes in the pungency of naturally pollinated ‘Shishito’ fruits. The present study newly revealed parthenocarpy-specific changes in several capsaicinoid biosynthesis genes, such as group 1 and group 2 genes, which were related to fluctuation in pungency levels. This provides new insight regarding the mechanism responsible for the occurrence of pungent ‘Shishito’ fruits. However, it is still unclear why a lack of seeds affects capsaicinoid biosynthesis or its accumulation. In the future, not only capsaicinoid biosynthesis, but also other metabolic processes should be investigated to clarify the mechanism using comprehensive transcriptome or metabolome analysis. In conclusion, we demonstrated that parthenocarpic induced fluctuations in the pungency of ‘Shishito’. ‘Shishito’ is a popular non-pungent chili pepper variety in Japan, and the present study is expected to add to the horticultural knowledge of ‘Shishito’ cultivation and controlling the pungency of fruits.

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