Involvement of Cytokinins, 3-Indoleacetic Acid, and Gibberellins in Early Fruit Growth in Pepper (*Capsicum annuum* L.)

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The roles of plant hormones in the early growth of pepper fruit (*Capsicum annuum* L.) were investigated. An exogenous hormone treatment study indicated that cytokinin (CK) was more effective at stimulating early fruit growth in two lines than auxin or gibberellin (GA). Endogenous levels of CKs, 3-indole-acetic acid (IAA), and GAs in young pollinated and unpollinated fruit of four lines (two with medium-sized and two with small fruit) were also investigated. In pollinated fruit, the level of *trans*-zeatin riboside (*tZR*) increased with fruit size. In unpollinated fruit, *tZR* did not increase in any lines. IAA levels decreased gradually after flowering and did not differ between pollinated and unpollinated fruit in any lines. Levels of GA₁ in unpollinated fruit of the lines in which unpollinated fruit were relatively well enlarged were slightly higher. In the line in which unpollinated fruit could not enlarge, GA₁ levels of all samples were lower than the others. These results indicate that *tZR* is important in the early enlargement of pollinated pepper fruit, and that GA₁ is involved in early fruit enlargement, especially in unpollinated pepper.

Key Words: forchlorfenuron, fruit enlargement, plant hormone, pollinate, unpollinate.

Introduction

Solanaceous vegetables such as tomatoes, eggplants, and peppers are autogamous plants with hermaphrodite flowers, and require pollination for fruit set and enlargement. A parthenocarpic trait, or a means to induce parthenocarpic fruit, which do not require pollination would be valuable for stable fruit production as these plants are sometimes cultivated under conditions unfavorable for pollination. In tomatoes (*Solanum lycopersicum* L.), many parthenocarpic genes have been reported, and some of them have been used in practical breeding (Gorguet et al., 2005). A method to improve fruit set by synthetic auxin treatment is well known and widely used in tomatoes and eggplants (Gillaspy et al., 1993; Gustafson, 1936). In tomatoes, auxin (mainly 3-indoleacetic acid: IAA) and gibberellins (GAs) play important roles in fruit set and enlargement (de Jong et al., 2009; Gillaspy et al., 1993).

In many pepper cultivars (*Capsicum annuum* L.), a low night temperature induces parthenocarpy (Rylski, 1973; Tarchoun et al., 2003; Tiwari et al., 2011). However, in such cultivars, the unpollinated parthenocarpic fruit set is lower than that of pollinated fruit, especially in the absence of low night temperatures. For example, in our previous study in ‘California Wonder’ (CW), for which parthenocarpic fruit set and enlargement have already been reported (Rylski, 1973), only 5% and 34% of fruit were set in unpollinated and pollinated conditions, respectively (Honda et al., 2012). In that study, fruit set between unpollinated and pollinated fruit among many pepper accessions was compared and it was reported that 40%, 80%, and 72% of ‘CNPH2622’ (CNP), ‘Shishitoh’ (SHI), and ‘INT/RUSSIA/2001/1579’ (INT) fruit were set in unpollinated conditions, while 30%, 94%, and 76% of those pollinated fruit were set, respectively. Within our experimental conditions, pollinated CW and CNP set medium-sized fruit (ca. 150 g and 100 g, respectively), whereas pollinated SHI and INT set small fruit (ca. 20 g). The parthenocarpic fruit of CNP and SHI were not enlarged.
Effects of hormone treatments and the physiological roles of hormones in fruit set and enlargement have been studied in pepper, although to a lesser extent than in tomatoes. IAA and GA treatments have been reported to enhance fruit set in pepper and to induce parthenocarpy in some studies (Heuvelink and Körner, 2001; Tiwari et al., 2012), but not in other studies (Bosland and Votava, 2012; Stover et al., 2000). No widely accepted method to improve fruit set and enlargement in pepper is available. Tiwari et al. (2013) reported that the endogenous IAA level increased at the late stage of fruit enlargement, but the endogenous GAs levels in pepper fruit have not yet been examined.

Another group of plant hormones, cytokinins (CKs), have been suggested to be involved in tomato fruit set and enlargement (Gillaspy et al., 1993); however, the role of CKs is less understood than those of IAA and GAs. Recent work in tomatoes suggests that CKs may regulate fruit growth (Ding et al., 2013; Mariotti et al., 2011; Matsuo et al., 2012). In pepper fruit, effects of CK treatment and endogenous CKs have not been examined.

The objective of our research was to examine the effects of plant hormones on pepper fruit enlargement in the early stage. First, the effects of exogenous plant hormone treatments on fruit growth were examined using pepper lines with small fruit (INT and SHI). Next, endogenous levels of CKs, IAA, and GAs at the early stage of fruit development were examined in these two lines and two lines with medium-sized fruit (CW and CNP). Comparative studies of different sizes and fruit enlargement characteristics may provide general information about the role of plant hormones in early fruit enlargement in pepper.

Materials and Methods

Plant materials

Pepper lines ‘INT/RUSSIA/2001/1579’ (INT) and ‘CNPH2622’ (CNP) were obtained from the collection of the NARO Institute of Vegetable and Tea Science (NIVTS). Cultivars ‘Shishitoh’ (SHI) and ‘California Wonder’ (CW) were obtained from Takii Seed Inc. (Kyoto, Japan). Fruiting characteristics of those lines are shown in Table 1.

Treatment with plant hormones

In the first treatment study, five plants each of INT and SHI were grown in summer–autumn 2009 in unglazed ceramic pots (24 cm diameter) containing a commercial soil mixture; plants were pruned to three or four stems. Healthy flowers at least 2 nodes apart (10–12 flowers per plant) were randomly selected 1 or 2 days before opening and the styles were excised to prevent pollination. Starting from 2 days after style excision, 100 ppm aqueous gibberellin A3 (GA3), 4-chlorophenoxyacetic acid (4-CPA), or forchlorfenuron (CPPU) (Wako Chemical, Osaka, Japan) solutions containing 0.1% methanol were spread on the ovaries and calyces evenly with a paint brush every second day over a 16-day period (8 applications). Control flowers were treated with solvent only. Three days after the last application, fruit were collected, measured and weighed, and the data were averaged. Pollinated fruit (not subjected to style excision) were also collected.

In the second treatment study, 10 plants were grown as described above in autumn–winter 2010–2011, and 6–8 flowers per plant were treated with 0.247, 2.47, or 24.7 ppm (1, 10, or 100 μM) CPPU as in the first treatment study.

All plants were grown and treated in a heated glasshouse at NIVTS at an average air temperature of 22.2°C (range, 16.3°C–34.4°C) in the first study and 18.5°C (2.8°C–31.0°C) in the second study.

Analysis of plant hormones

Eight plants each of CW, CNP, INT, and SHI were grown in spring–summer 2010 as described above. From 3 to 12 June, healthy flowers were selected 1 or 2 days before opening, styles were excised, and the date of flowering was recorded. On 14 June, all fruit including calyces were collected, weighed, frozen in liquid nitrogen, and stored at –80°C. We also collected pollinated fruit without style excision and flowers with calyces on the day of opening. All samples were classified according to the number of days after flowering (DAF) and treatment. All plants were grown in the glasshouse at NIVTS (average air temperature 26.2°C, range 17.0°C–41.2°C).

| Line   | Fruit size | Fruit setting rate of fruit | Enlargement of unpollinated fruit |
|--------|------------|-----------------------------|----------------------------------|
| CW     | medium     | medium                      | low                              | slightly enlarge               |
| CNP    | medium     | medium                      | medium                           | well enlarge, not enough       |
| INT    | small      | high                        | high                             | not enlarge                    |
| SHI    | small      | high                        | high                             | well enlarge, not enough       |

Table 1. Fruiting characteristics of the peppers used in this study.
Flowers and 3, 6, and 9 DAF fruit were homogenized in 80% methanol and filtered. The following internal standards were added to extracts corresponding to 1–2 g fresh weight: 5 ng each of [3H]-CK ([3H]-trans-zeatin, [3H]-trans-zeatin riboside, [3H]-d, l-dihydrozeatin, [3H]-trans-(R,S)-dihydrozeatin riboside, [3H]-N\textsubscript{6}-(2-isopentenyl)adenine, and [3H]-N\textsubscript{6}-(2-isopentenyl)adenosine; all from Apex Organics, Exeter, UK), 10 ng of [13C\textsubscript{5}]-IAA (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA), and 5 ng each of [2H\textsubscript{2}]-GA ([2H\textsubscript{2}]-GA\textsubscript{4}, [2H\textsubscript{2}]-GA\textsubscript{5}, and [2H\textsubscript{2}]-GA\textsubscript{20}, all from Olchémim, Olomouc, Czech Republic). The extracts were evaporated to dryness. The residues were run in positive ion mode for CKs and IAA and negative ion mode for GAs using a Turbo V ion source operated in ESI mode (AB Sciex, Framingham, MA, USA). The mass spectrometer was coupled to a 3200 Q TRAP linear ion trap quadrupole mass spectrometer equipped with a JMS-BU-20 mass spectrometer (JEOL, Tokyo, Japan) coupled to a 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) according to Shiraiwa et al. (2012).

### Results

**Effects of plant hormone treatments**

Pollinated INT and SHI fruit were a similar size and shape (ca. 20 g, 5–7 cm long, 1–1.5 cm wide). Unpollinated fruit of INT did not enlarge, but that of SHI showed slight enlargement.

INT fruit were significantly wider after CPPU treatment than after other treatments or pollination (Fig. 1A). INT fruit length after CPPU treatment was similar to that of pollinated fruit, but was significantly greater than that in unpollinated controls, whereas the length of GA\textsubscript{3} and 4-CPA-treated fruit did not differ significantly from that of controls (Fig. 1B). The weight of fruit treated with CPPU was similar to that of pollinated fruit and significantly greater than those of other fruits (Fig. 1C). Control fruit never enlarged even if they were located at a node close to a node where hormonally treated fruit was located, indicating that hormonal treatment of a particular fruit did not affect the enlargement of other fruits.

The width (Fig. 1D) and weight (Fig. 1F) of SHI fruit treated with CPPU were significantly greater than those in other treatments and pollinated fruit. Effects of GA\textsubscript{3} and 4-CPA on SHI were similar to those on INT. After CPPU treatment, SHI fruit were significantly longer than those in controls, but did not differ significantly from those after other treatments or pollinated fruit (Fig. 1E).

Among 10–12 INT fruit, 3 fruit abscised in controls, 4 after GA\textsubscript{3} treatment, and 1 after CPPU treatment, whereas no SHI fruit abscised.

In the second treatment study, which used three lower concentrations of CPPU, the weight of INT fruit treated with 24.7 ppm (100 μM) CPPU was similar to that in pollinated fruit and was significantly greater than that in controls, whereas that after 0.247 or 2.47 ppm (1 or 10 μM) CPPU treatment was slightly, but not significantly, higher than that of the controls (Fig. 2A). SHI showed a similar tendency (Fig. 2B), but the differences were not statistically significant. No fruit abscised in either line.

**Fruit growth**

Unpollinated fruit of CW, CNP, and SHI with excised styles grew more slowly than corresponding pollinated fruit (Fig. 3), and those of CNP and SHI were relatively well enlarged compared to that of CW (65%, 49%, and 36% of the corresponding pollinated fruit at 10 DAF, respectively). In pollinated fruits of all four lines, the fastest weight increase was observed around 9 DAF. Unpollinated fruit of INT did not enlarge (Fig. 3C). Pollinated and unpollinated fruit at 3, 6, and 9 DAF and flowers at the opening stage were selected from these plants and used to determine the levels of CKs, IAA, and GAs.
Analysis of CKs

Three endogenous CKs, trans-zeatin (tZ), trans-zeatin riboside (tZR), and isopentenyladenosine (iPR) were identified. CKs such as cis-zeatin (cZ), cis-zeatin riboside (cZR), dihydrozeatin (DZ), dihydrozeatin riboside (DZR), or isopentenyladenine (iPA) were not identified in any samples. The levels of tZ were generally decreased in all lines and the pattern of changes did not clearly differ between pollinated and unpollinated conditions (Fig. 4). The levels of tZR in pollinated fruit increased to 9 DAF except in CNP, in which it peaked at 6 DAF, but those increases were not observed in unpollinated fruit. The maximum amounts of tZR except for CW were significantly higher than those of other fruit samples, and that of SHI was particularly marked. In unpollinated INT fruit, tZR was not detected in every
sample. The levels of iPR also decreased in all lines and did not clearly differ between pollinated and unpollinated conditions.

Analysis of IAA

The levels of IAA gradually decreased, and did not clearly differ between pollinated and unpollinated fruit in any lines (Fig. 5). The IAA levels in INT and SHI were higher than those in CW and CNP.

Analysis of GAs

In our preliminary analysis of endogenous GAs in pepper fruit, GA$_1$, GA$_{20}$, and GA$_{19}$ were identified, but GA$_4$, GA$_9$, or GA$_{24}$ were not. Then, the levels of GA$_1$, GA$_{20}$, and GA$_{19}$ were examined. The patterns of GA changes differed among the four lines (Fig. 6). As described earlier, unpollinated fruit of CNP enlarged relatively well compared to that of CW. In CW, the levels of all GAs in pollinated fruit at 3 DAF were significantly higher than those in flowers and unpollinated fruit at 3 DAF. GA$_{20}$ was not detected in flowers or unpollinated fruit at any time point, and GA$_1$ was not detected in unpollinated fruit at 9 DAF (Fig. 6A). In CNP, the GA$_1$ level of flowers at 3 and 6 DAF both in pollinated and unpollinated fruit did not significantly differ. GA$_{20}$ was detected in all CNP samples except flowers (Fig. 6B). In INT, in which unpollinated fruit did not enlarge, GA$_1$ was not detected in pollinated fruit at 3 or 6 DAF or in unpollinated fruit at 6 or 9 DAF, and was present at lower levels in other INT samples than in CW, CNP, and SHI. GA$_{20}$ was not detected in unpollinated INT fruit at 3 or 6 DAF (Fig. 6C). In SHI, in which unpollinated fruit enlarged relatively well compared to that of CW, GA$_1$ and GA$_{20}$ were not detected in flowers, but were detected in all fruit samples, and there were no clear pollination-dependent differences (Fig. 6D). Levels of GA$_1$ in INT and SHI flowers were lower than those of CW and CNP. GA$_{19}$ was detected in all samples and the levels in 3 DAF pollinated fruit were higher than those of corresponding flowers except for CNP, while the levels decreased gradually in pollinated fruit except for CNP.

Discussion

To clarify the roles of CKs, IAA, and GAs in early fruit development in pepper, the effects of exogenous treatment and endogenous levels of these three hormones were examined.

The role of CKs

In INT, untreated unpollinated fruit never enlarged, but treatment with 100 ppm CPPU, a synthetic CK, enlarged INT fruit. CPPU treatment also enlarged SHI fruit (Fig. 1). The effects of CPPU were gradually decreased with decreases in the applied CPPU concentration (Fig. 2). The greater effect of CPPU than of GA$_3$ and 4-CPA, and the dose-dependent effect, indicated the important role of CK in early fruit enlargement (Fig. 1).

Using immunoaffinity purification, Ulvskov et al. (1992) identified tZ as a CK base and tZR and iPR as
CK ribosides, but there have been no other reports on endogenous CKs in pepper. In this study, no other CKs (cZ, cZR, DZ, DZR, or iPA) were identified in any samples. These results indicate that tZ, tZR, and iPR are predominant in pepper fruit. The highest tZR levels were detected in pollinated fruit of CW, INT, and SHI at 9 DAF, when the fastest fruit weight increase was observed, indicating that tZR levels are related to fruit weight increases (Figs. 3 and 4). However, in pollinated CNP fruit, tZR was highest at 6 DAF; this may indicate a difference in the fruit enlargement mechanism between CNP and other lines examined. In unpollinated fruit, there was no similar increase in tZR in any pepper line (Fig. 4). Using ‘Pearson’ tomatoes, Mariotti et al. (2011) showed that the levels of CK ribosides (tZR, DZR, and iPR) and iPA were highest at anthesis and then clearly decreased, whereas those of tZ and DZ were highest 5 days after anthesis; the levels of tZ and DZ were lower than those of tZR, which was the highest among the six CKs. CPPU treatment had dose-dependent effects on ‘Micro-Tom’ fruit enlargement (Matsuo et al., 2012). These results for tomatoes are generally similar to our results for pepper. In an assay to examine CK binding to Arabidopsis CK receptors, tZR showed one-tenth of the affinity of tZ with the CRE1/AHK4 receptor, but had the same affinity as tZ with the AHK3 receptor, indicating that tZR has genuine biological activity (Spíchal et al., 2004). Therefore, the tZR increase with fruit enlargement may indicate that tZR plays an important role in early enlargement of pollinated pepper fruit.

**The role of IAA**

Synthetic auxin treatment showed slight, but not significant, positive effects on fruit length and weight.
Fig. 5. Endogenous IAA in pepper fruit. A, ‘California Wonder’; B, ‘CNPH2622’; C, ‘INT/RUSSIA/2001/1579’; D, ‘Shishitoh’. Error bars indicate SE. Significance of the differences was analyzed by compound and line. Different letters above bars show significant differences (Tukey-Kramer test, \(P < 0.05\)). Three independent biological replicates were analyzed except for pollinated CW at 6 DAF, for which two samples were analyzed.

Fig. 6. Endogenous gibberellins in pepper fruit. A, ‘California Wonder’; B, ‘CNPH2622’; C, ‘INT/RUSSIA/2001/1579’; D, ‘Shishitoh’. Black bars, GA\(_1\); shaded bars, GA\(_{20}\); white bars, GA\(_{19}\). Error bars indicate SE. Significance of the differences was analyzed by compound and line. Different letters above bars show significant differences (Tukey-Kramer test, \(P < 0.05\)). Three independent biological replicates were analyzed except for pollinated CW at 6 DAF, for which two samples were analyzed. *Not detected and not compared to other data.
IAA levels generally decreased and did not differ between pollinated and unpollinated fruit (Fig. 5) in any lines.

In peppers, Tiwari et al. (2013) found that IAA levels decreased gradually for up to 12 days after anthesis and then increased 15–20 days after anthesis in carpels. In unpollinated fruit, similar levels of IAA were detected in the early stage of fruit development and also gradually decreased for up to 12 days after anthesis, but did not increase in the subsequent days. On the basis of these results, Tiwari et al. (2013) concluded that IAA also plays an important role in the later stages of fruit growth. High IAA levels in the early stage and their subsequent decline were also shown in tomatoes (Mariotti et al., 2011). In tomatoes, the positive effects of synthetic auxins such as 4-CPA on the induction of parthenocarpic fruit set and growth are well known (Gillaspy et al., 1993). In pepper, IAA may not be important or very limited in the early stage of fruit growth, and its role is not affected by pollination.

**The role of GAs**

Treatment with GA$_3$ slightly, but not significantly, increased fruit length and weight. Biosynthesis of bioactive GAs in higher plants mainly occurs via two pathways: the early C13-hydroxylation pathway produces GA$_1$, whereas the non-C13-hydroxylation pathway produces GA$_4$; the occurrence of these pathways differs among plant species and organs (MacMillan, 1997, 2002). As described above, only three endogenous GAs (GA$_1$, GA$_{20}$, and GA$_{19}$) produced in the early C13-hydroxylation pathway were identified in pepper fruit.

In a study of the effects of GA-producing rhizobacteria in red pepper seedlings, Joo et al. (2005) identified GAs produced by both pathways and reported that the non-C13-hydroxylation pathway was predominant. There are no other reports of endogenous GAs in peppers. In tomatoes, despite a large number of studies, GA$_4$ has not been identified in fruit, but has been in seedlings (MacMillan, 2002). These results suggest that the dominant GA biosynthesis pathway in pepper fruit is the early C13-hydroxylation pathway that produces bioactive GA$_1$ through GA$_{20}$ and GA$_{19}$; therefore, the levels of GA$_1$, GA$_{20}$, and GA$_{19}$ were examined.

The differences in the patterns of GA$_1$ changes among the lines suggest that GA$_1$ may be related to fruit phenotypes. In CNP, GA$_1$ levels of both 3 DAF fruits were similar, but that of unpollinated CW was lower than that of the pollinated one (Fig. 6A, B). Unpollinated fruit of CNP grew better than that of CW (Fig. 3A, B). There were no clear differences in IAA and CK levels between CW and CNP (Figs. 4A, B and 5A, B). These results may indicate that GA$_1$ relates to faster growth of unpollinated CNP fruit.

Higher GA$_{20}$ levels in pollinated than in unpollinated fruit of CW, CNP, and INT (Fig. 6) may indicate that GA-20-oxidase (GA20ox), which catalyzes biosynthes-

sis of GA$_{20}$ from GA$_{20}$, through GA$_{24}$ and GA$_{19}$ (MacMillan, 1997), is activated by pollination. In SHI, levels of GA$_{1}$ and GA$_{20}$ were not clearly different between pollinated and unpollinated fruit. These results indicate that GA20ox was activated in SHI even in unpollinated fruit and that this may contribute to considerable active GA$_1$ levels and enlargement of unpollinated fruit. In INT, although GA$_{20}$ increased, GA$_1$ was not detected in 3 and 6 DAF pollinated fruits (Fig. 6C). These results may indicate a certain defect in GA-3-oxidase (GA3ox), which catalyzes biosynthesis of GA$_1$, from GA$_{20}$ (MacMillan, 1997) in INT; and this defect may be related to the failure of unpollinated INT fruit to enlarge. The IAA level was higher in INT than in CNP and CW (Fig. 5). In INT, tZR clearly increased in pollinated fruit, but was not detected in unpollinated fruit (Fig. 4C).

All these results in GAs indicate that GA$_1$ plays an important role in the early stage of fruit enlargement, especially in unpollinated conditions when tZR is lower or not present.

In tomatoes, the role of GAs in fruit set and early fruit growth is well characterized. Similarly to auxins, GAs can induce parthenocarpic fruit set, and GA levels in the ovaries of parthenocarpic lines are higher than those of normal lines (Fos et al., 2000; Olimpieri et al., 2007). The expression of the GA20ox gene is higher in the ovaries of parthenocarpic lines than in those of normal tomato lines (Olimpieri et al., 2007). Auxin-induced parthenocarpic tomato fruit contains high levels of transcripts for the GA biosynthetic enzymes GA20ox and GA3ox and high levels of GAs (Mariotti et al., 2011; Serrani et al., 2008). Our results on the role of GA$_1$ in pepper in early fruit growth are similar to those obtained in tomatoes.

**Role of plant hormones in fruit set**

As fruit abscission is very limited even in controls (unpollinated fruit with no treatment), the effect of treatments on fruit set could not be observed in this study, and the role of CKs in fruit set in peppers remains unknown. Our data showing high CKs levels in flowers may indicate a positive role of CKs during fruit set. Ding et al. (2013) showed that CPPU treatment increased fruit set in a dose-dependent manner in ‘Micro-Tom’.

As described earlier, the fruit setting rates of CW and CNP were lower than those of INT and SHI. Higher levels of IAA in the flowers of INT and SHI than those of CW and CNP suggests a positive relation with IAA in fruit set, but further studies are needed to confirm this.

**Conclusion**

Our data show that the levels of GAs and particularly CKs, but not those of IAA, are changed dramatically by pollination. A clear increase in the levels of tZR in pol-
linated fruit may indicate its importance in early pepper fruit enlargement. There is no practical method to improve or manage fruit set in peppers. The fruit set rate is lower in pepper lines with larger fruit, and a method to increase fruit set is needed. Therefore, the effects of CK-related compounds such as CPPU on pepper fruit set are of interest, and further studies on lines with large fruit are needed. The basic knowledge obtained from the data presented here may contribute to future studies aimed at unveiling the mechanism of fruit set and enlargement not only in peppers, but also in other solanaceous vegetables such as tomatoes and eggplants.

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