Seed Production Enhanced by Antiauxin in the *pat-2* Parthenocarpic Tomato Mutant

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ABSTRACT. There is concern that high temperatures resulting from global warming could reduce fruit set of tomato (*Solanum lycopersicum*). However, fruit set of parthenocarpic tomato genotypes, which often bears seedless fruit, is not reduced when grown under a high temperature. The cause of seedless fruit development was studied with the aim of increasing the seed number in parthenocarpic tomato. Ovule number at anthesis in parthenocarpic and non-parthenocarpic fruit did not differ, but the proportion of undeveloped ovules increased with time after anthesis in parthenocarpic tomato, whereas most ovules in non-parthenocarpic tomato developed normally. Pollen grains germinated on the stigma and extruded pollen tubes in parthenocarpic and non-parthenocarpic tomatoes, but in parthenocarpic tomato, pollen tube elongation was markedly inhibited in the style base. Elongation of pollen tubes on agar containing indoleacetic acid (IAA) was depressed in parthenocarpic and non-parthenocarpic tomato plants. *p*-Chlorophenoxyisobutyric acid (PCIB), which inhibits auxin action, did not affect the fruit set and fresh weight in either type of tomato, although seed number per fruit in parthenocarpic tomato was significantly increased from 13 ± 2 to 74 ± 6 seeds by PCIB treatment. These results indicated that a high IAA concentration in the ovary of parthenocarpic tomato inhibited pollen tube elongation, and that poor fertilization resulted in failure of ovule development. Moreover, floral organs in parthenocarpic tomato were normally developed as in non-parthenocarpic tomato, and seed development could be induced in parthenocarpic tomato by PCIB treatment.

Global warming threatens agricultural production. In plants, reproductive development is more sensitive to high temperature stress than vegetative development, and the sensitivity to high temperature stress appears to differ among crops (Sato et al., 2006). In tomato, the optimal temperature for fruit set was reported as 21 to 24 °C (Geisenberg and Stewart, 1986) and 22 to 25 °C (Peet and Bartholomew, 1996). In addition, Sato et al. (2002, 2006) reported that pollen viability and release were affected adversely by high temperature and became major limiting factors for fruit set in tomato.

In general, the development of fruit occurs following pollination and fertilization, and coincides with maturation of the seeds (Gorguet et al., 2005). Parthenocarpy is the phenomenon by which fruit set is induced by plant growth regulators, mainly auxin and gibberellins (GAs), without pollination and fertilization, and parthenocarpic fruit can develop in harsh environments (Srivastava and Handa, 2005). In non-parthenocarpic tomato, failure of fruit set occurs under unfavorable environmental conditions such as high temperatures (George et al., 1984). Therefore, a tomato cultivar with the parthenocarpic phenotype has the potential for achieving improved yields under global warming.

Genetic parthenocarpy in tomato is the result of expression of the *pat*, *pat-2*, or *pat-3/pat-4* genes (Ficcadenti et al., 1999; Fos and Nuez, 1996, 1997; George et al., 1984; Gorguet et al., 2005; Lukyanenko, 1991; Mazzucato et al., 1998). The parthenocarpic tomato underlying *pat* genes have been studied, namely development of flower tissue with *pat* (Mazzucato et al., 1998, 2003) or *pat-2* genes (Kataoka et al., 2003), and the role of endogenous hormones with *pat-2* (Fos et al., 2000; Kataoka et al., 2004) or *pat-3/pat-4* (Fos et al., 2001), or polyamines with *pat-2* (Fos et al., 2003). Seedless fruit or fruit with fewer seeds in tomato was induced genetically (Fos et al., 2000, 2001; Mazzucato et al., 1998) or artificially, that is, auxin and GAs (Hamamoto et al., 1998; Koshioka et al., 1994; Serrani et al., 2007), but the cause of seedless or fewer seeds is not clear. Elucidation of the seedless mechanism is necessary to produce seeds of parthenocarpic cultivars for their sexual reproduction.

Fertilization requires pollen germination, and penetration and elongation of pollen tubes in stylar tissue toward the embryo sac for double fertilization to occur. Mazzucato et al. (2003) reported that pollen tubes appeared to become disoriented in the ovarian cavity and often failed to adhere to the placental surface in the *pat* tomato mutant. It has been suggested that parthenocarpy may be the result of elevated hormone levels in the ovary even in the absence of pollination and fertilization (Gillaspy et al., 1993; Nitsch, 1970). Our hypothesis was that a high concentration of endogenous auxin

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in the parthenocarpic ovary may inhibit pollen tube growth in the lower style and thus prevent fertilization. Therefore, we investigated pollen tube elongation in the style and ovule development in the ovary of *pat-2* parthenocarpic tomato. In addition, the effect of a range of concentrations of auxin (IAA) on pollen tube elongation was studied. The results contribute to our understanding of the mechanism controlling seed number in *pat-2* parthenocarpic tomato.

Materials and Methods

PLANT MATERIALS. A parthenocarpic tomato carrying the *pat-2* gene, cultivar Renaissance (Sakata Seed, Yokohama, Japan) that has large fruit and was readily available from commercial sources and a non-parthenocarpic tomato, cultivar Momotaro (Takii Seed, Kyoto, Japan) that also has large fruit were used in the experiments.

Tomato seeds of each cultivar were pregerminated for 3 d at 25 °C under dark conditions. Germinated seeds were sown in a 72-cell tray (50 mL per cell) filled with a mixture of peatmoss and vermiculite (2:1, v/v). At 30 d after sowing, the seedlings were transplanted to 6-cm-diameter plastic pots filled with the same substrate, and were transplanted again to 20-cm-diameter pots at 50 d after sowing. The plants were grown in a greenhouse at Osaka Prefecture University (Sakai, Japan). A nutrient solution containing (in mmol·L⁻¹) 4.6 N (4.1 NO₃⁻, 0.4 NH₄⁺), 1.3 P, 2.2 K, 1.1 Ca, and 0.4 Mg was applied from April to October and 9.2 N (8.2 NO₃⁻, 0.8 NH₄⁺), 2.6 P, 4.4 K, 2.2 Ca, and 0.8 Mg was applied from November to March. The plants were staked and all lateral shoots were removed while less than 1 cm in length.

OVULE DEVELOPMENT AFTER POLLINATION. All tomato seeds were sown on 20 Aug. 2004 and cultivated at 33.4/24.5 °C (maximum/minimum temperature) in the greenhouse. Five to 10 flowers per plant that opened between 13 and 15 Oct. were emasculated before anthesis, and five flowers per plant were artificially self-pollinated at anthesis. Twenty self-pollinated ovaries were sampled 0, 1, 2 and 3 weeks after anthesis, respectively, and were fixed, dehydrated, and stained according to Johkan et al. (2008). Ovary transverse sections (12 or 20 µm thick) were observed by light microscopy (BX-50; Olympus, Tokyo), and the number of ovules were counted in 15 ovaries. Seed number and fruit fresh weight in 30 mature fruit were also measured.

POLLEN TUBE ELONGATION IN STYLE. All tomato seeds were sown on 16 Apr. 2004 and cultivated at 28.9/16.4 °C (maximum/minimum temperature) in the greenhouse. Five to 15 flowers per plant that opened between 5 and 7 June were emasculated before anthesis, and five flowers per plant were artificially pollinated. Twenty styles were sampled 72 h after pollination and were then fixed. After fixation, the styles were embedded in an 8% agar block. Transverse sections 200 µm thick, each including the four style sections distinguished (Fig. 1), were prepared with a vibrating blade microtome (VIB-1500; Vibratome, St. Louis), stained with aniline blue, and observed with fluorescence microscopy (BX-50; Olympus). Areas of the pollen tube in five styles per three replications were digitized with Image software (Scion, Frederick, MD), and the arrival rate of pollen tubes in each style region was expressed as a percentage of that in the S1 area (Fig. 1).

EFFECT OF INDOLEACETIC ACID ON POLLEN TUBE ELONGATION. All tomato seeds were sown on 23 Aug. 2006 and cultivated at 33.1/15.1 °C (maximum/minimum temperature) in the greenhouse. Anthers per 10 flowers were sampled on 24 Oct. and dried overnight. Pollen grains were incubated on 0.8% agar containing 3% sucrose and 0, 0.1, 1, or 10 mg·L⁻¹ IAA at 25 °C, and 10 pollen tube length in five replications was observed by light microscopy (BX-50; Olympus).

EFFECT OF *P*-CHLOROPHENOXYISOBUTYRIC ACID ON SEED SET IN PARTHENOCARPC FRUIT. All tomato seeds were sown on 4 Sept. 2007 and cultivated at 32.4/16.6 °C (maximum/minimum temperature) in the greenhouse. Five to 10 flowers of the parthenocarpic tomato were dipped in 0 or 100 mg·L⁻¹ PCIB 7 d before anthesis. The PCIB-treated flowers that opened between 3 and 10 Nov. were emasculated before anthesis and five flowers per plant were artificially pollinated. Fruit set per all pollinated flower, fruit fresh weight, seed set per all fruit and seed numbers in all fruit contained with seeds and without seeds were counted in 30 mature fruit.

STATISTICAL ANALYSIS. The difference between two independent means was tested with Student's *t* test at the 0.05, 0.01, and 0.001 significance levels. Tukey's multiple range tests were used to test the difference between more than two means at the 0.05 significance level using statistics software (MEPHAS; Osaka University, Suita, Japan).

Results

OVULE DEVELOPMENT AFTER POLLINATION. The average numbers of ovules per fruit at anthesis and fruit fresh weight were not significantly different between the parthenocarpic and

Fig. 1. Definition of S1 to S4 regions of the style in tomato. Four regions in the style were recognized to evaluate pollen tube elongation in the style.

Table 1. Fruit fresh weight and number of ovules and seeds per fruit in non-parthenocarpic and parthenocarpic tomato plants.

Type	Fruit fresh wt [mean ± SE (g)]	Ovules [mean ± SE (no./fruit)]	Seeds [mean ± SE (no./fruit)]
Non-parthenocarpic	211 ± 3	202 ± 7	191 ± 1
Parthenocarpic	207 ± 1	173 ± 1	31 ± 5
<i>t</i> test	NS	NS	***

*** different at 0.001 significance level as indicated by Student's *t* test; NS = not significantly different at 0.05 level.

non-parthenocarpic tomato cultivars (Table 1). However, the number of seeds per fruit was significantly lower in parthenocarpic tomato than that in non-parthenocarpic tomato, whereas fruit fresh weight was not significantly different between the cultivars.

To investigate the inhibition of seed development in parthenocarpic tomato, ovules were observed at various developmental stages in parthenocarpic and non-parthenocarpic tomato (Fig. 2). The ovules in both types of tomato were normal at 0 and 1 week after anthesis (Fig. 2, A–D). At 2 and 3 weeks after anthesis, undeveloped ovules were observed in parthenocarpic tomato (Fig. 2, F and H), whereas developing ovules were present in non-parthenocarpic tomato fruit (Fig. 2, E and G).

Ovule development was distinctly arrested in parthenocarpic tomato (Fig. 3). One week after anthesis, the proportion of developing ovules in parthenocarpic tomato was similar to that of non-parthenocarpic tomato. Two weeks after anthesis, however, the proportion of developing ovules in parthenocarpic tomato was significantly reduced to 55% compared with 94% in non-parthenocarpic tomato, and the proportion further declined to 24% in parthenocarpic tomato 3 weeks after anthesis.

Fig. 2. Transverse sections of developing ovules in non-parthenocarpic (left) and parthenocarpic (right) tomato. Images are of ovaries at 0 weeks (A and B), 1 week (C and D), 2 weeks (E and F), and 3 weeks (G and H) after anthesis. do = developing ovule, uo = undeveloped ovule.

POLLEN TUBE ELONGATION IN STYLE. At 72 h after pollination, penetration of pollen tubes in the style transmitting tissue in parthenocarpic and non-parthenocarpic tomato was examined by fluorescent microscopic observation (Fig. 4). Style in tomato has 3 to 5 transmitting tissue in the septum irrespective of genotype (Fig. 4). Pollen tubes were observed in transmitting tissue in the S1 (Fig. 4, A and B) and S4 (Fig. 4, C and D) style regions.

Arrival rate of pollen tubes in the S2 style region (Fig. 1) did not differ between parthenocarpic and non-parthenocarpic tomato (Fig. 5). However, only 21% of pollen tubes reached the S3 region in parthenocarpic tomato, which differed significantly from the high arrival rate (72%) of pollen tubes in the S3

Fig. 3. Proportion of developed ovules in non-parthenocarpic and parthenocarpic tomato; ** indicates significance at $P = 0.01$ by Student's t test.

Fig. 4. Pollen tubes in style of non-parthenocarpic (left) and parthenocarpic (right) tomato 72 h after pollination. Transverse sections of the S1 (A and B) and S4 (C and D) regions of the style were stained with aniline blue and viewed with fluorescence microscopy. pt = pollen tube, vb = vascular bundle, tt = transmitting tissue, bars = 100 μ m.

Fig. 5. Arrival rate of pollen tubes in different regions (S1–S4) of the style in non-parthenocarpic and parthenocarpic tomato 72 h after pollination; * and ** indicate significance at $P = 0.05$ and 0.01 , respectively, by Student's t test.

Fig. 6. Effect of IAA concentration on pollen grain germination in non-parthenocarpic and parthenocarpic tomato. Pollen grains were germinated on 0.8% agar containing IAA. The rate of pollen germination was measured 24 h after sowing. Different letters indicate a significance difference at $P = 0.05$ with Tukey's multiple range tests.

region in non-parthenocarpic tomato. Only 10% of pollen tubes in parthenocarpic tomato reached the S4 region at the style base.

EFFECT OF INDOLEACETIC ACID ON POLLEN TUBE ELONGATION. At 24 h after sowing, the rate of pollen germination decreased with increasing IAA concentration in both types of tomato (Fig. 6).

The mean pollen tube length was 150 μm in the absence of IAA but was significantly reduced by IAA in both tomato types 12 h after sowing (Fig. 7). Pollen tube elongation was increasingly inhibited with increasing IAA concentration ($\geq 1 \text{ mg}\cdot\text{L}^{-1}$).

EFFECT OF P-CHLOROPHOXYISOBUTYRIC ACID ON SEED SET IN PARTHENOCARPCIC FRUIT. PCIB treatment did not affect the rate of fruit set (100%) and the mean fruit fresh weight in parthenocarpic tomato (Table 2). PCIB treatment increased

Fig. 7. Effect of IAA concentration on pollen tube elongation in non-parthenocarpic (○) and parthenocarpic (●) tomato. Pollen grains were sown on 0.8% agar containing IAA. Different letters above symbols at each time point indicate a significant difference at $P = 0.05$ with Tukey's multiple range tests.

seed set per fruit in parthenocarpic tomato from 83% to 92%, and the seed number per fruit was significantly increased from 13 ± 2 to 74 ± 6 seeds. The greater seed number (mean of 74 seeds) of parthenocarpic tomato in response to PCIB treatment was almost one-half the seed number (mean of 152 seeds) of non-parthenocarpic tomato.

Discussion

In most fruit, normal ovary growth requires the presence of seeds (Nitsch, 1970), and seeds play an important role in normal fruit development (Srivastava and Handa, 2005). Fruit fresh weight in non-parthenocarpic tomato was proportional to the number of seeds per fruit (Groot and Karssen, 1987) because development of the ovary requires plant growth regulations originating from the seeds (Sponsel, 1983; Talon et al., 1990; Rodrigo et al., 1997). In parthenocarpic tomato, the ovary is able to develop without ovule fertilization because the ovary synthesizes high levels of plant growth regulation (Fos et al., 2000, 2001; George et al., 1984; Mapelli et al., 1978; Olimpieri et al., 2007; Pandolfini et al., 2002), and few or no seeds were formed in fruit of normal fresh weight (Carmi et al., 2003). However, the number of ovules at anthesis did not differ between parthenocarpic and non-parthenocarpic tomato in our experiment. These results indicated that developmental arrest of ovules might account for the absence of seed maturation in parthenocarpic tomato.

In the present study, ovule morphology in the parthenocarpic tomato was normal at anthesis, but most ovules failed to develop after pollination. In the *pat* tomato mutant, the majority of ovules remained undeveloped and failed to increase in size after anthesis, and a fraction of ovules became aberrant from very early stages before anthesis (Mazzucato et al., 1998). Moreover, pollen tubes in *pat* and *pat-2* tomato were disorientated in the ovarian cavity and often failed to adhere to the placental surface (Mazzucato et al., 2003). Mazzucato et al. (2003) considered that disorientated pollen tubes in the ovarian cavity were caused by aberrant ovules, which not only lack attractiveness, but disrupt pollen tube guidance overall. In this study, aberrant ovules were

Table 2. Effect of *p*-chlorophenoxyisobutyric acid (PCIB) on fruit set, fruit fresh weight, and seed set and seeds number per fruit in parthenocarpic and non-parthenocarpic tomato plants.

Type	PCIB conc (mg·L ⁻¹)	Fruit set ^z (%)	Fruit fresh wt [mean ± SE (g)]	Seed set ^y (%)	Seeds [mean ± SE (no./fruit)]
Non-parthenocarp	—	87	180 ± a ^x	100	152 ± a
Parthenocarp	0	100	167 ± a	83	13 ± c
	100	100	164 ± a	92	74 ± b

^zFruit set was calculated by fruit per artificially pollinated flower.

^ySeed set was calculated by fruit with seed per all fruit.

^xSame letters in each column indicate no difference with Tukey's multiple range test at the 0.05 significance level.

not observed at anthesis, but disorientated pollen tubes, such as those in the *pat* tomato mutant, might occur also in the parthenocarpic tomato with *pat-2*.

Low seed set in *Cornus florida*, *Hydrangea paniculata*, and *Hydrangea quercifolia*, which are self-incompatible, was induced by inhibition of pollen tube elongation (Reed, 2004a, 2004b). In tetraploid grape (*Vitis vinifera*), poor set of seeded berries in most tetraploid grapes was due to poor development of transmitting tissue in the septum, which severely inhibited pollen tube penetration into the micropyle (Okamoto et al., 2001). In this study, the morphology of transmitting tissue was no different between parthenocarpic and non-parthenocarpic tomato. In addition, seed development in *pat-2* parthenocarpic tomato was affected by the maternal parent irrespective of the pollen parent, and the seeds tended to remain undeveloped when the ovary parent was parthenocarpic (Ohkawa et al., 2008). Because pollen tube elongation in the style was inhibited near the style base in parthenocarpic tomato, it is conceivable that a factor induces inhibition of pollen tube elongation close to the style base in parthenocarpic tomato.

Auxin plays an important role in the development of parthenocarp (Gorguet et al., 2005). The expression of genetic parthenocarp is correlated with the accumulation of auxins in the ovaries, which is autonomous and precocious compared with the respective wild types (George et al., 1984; Ikeda et al., 1999; Mapelli et al., 1978; Pandolfini et al., 2002). It was considered that the concentration of the endogenous auxin, IAA, at the base of the style may be higher than that near the stigma, and a high level of IAA may inhibit the growth of pollen tubes in parthenocarpic tomato. In our experiment, pollen tube elongation was inhibited by at least 0.1 mg·L⁻¹ IAA. The concentration of IAA in *pat* mutant parthenocarpic and non-parthenocarpic tomato fruit at anthesis was 0.16 and 0.05 mg·kg⁻¹ fresh weight, respectively (Mapelli et al., 1978).

In our experiment, PCIB, which is described as an antiauxin or an auxin antagonist (Foster et al., 1995; Kim et al., 2000) and inhibitor of auxin action by binding to the auxin receptor site (MacRae and Bonner, 1953), increased the number of seeds in parthenocarpic tomato. Moreover, 2,3,5-triiodobenzonic acid, which is considered an antiauxin (Katayama and Marumo, 1994) or inhibitor of auxin transport (Christie and Leopold, 1965), increased the IAA concentration in the ovary and reduced the number of seeds in non-parthenocarpic tomato (Hamamoto et al., 1998). These results indicated that a reduced concentration of IAA in the ovary of parthenocarpic tomato in response to PCIB treatment allowed seed set, and therefore, the ovules in parthenocarpic tomato were normally developed, similar to those of non-parthenocarpic tomato. As parthenocarpic tomato formed pseudoembryos, which formed in the ovule cavity

(Kataoka et al., 2003), and aberrant ovules (Mazzucato et al., 2003), abnormal or arrested development of ovules in parthenocarpic tomato may be caused by autointoxication of auxin.

The number of seeds in parthenocarpic tomato was promoted by PCIB treatment, but was still less than that of non-parthenocarpic tomato. Fos et al. (2000) reported that parthenocarp induced by the *pat-2* gene was a response to increased synthesis of active GAs. The concentration of GAs in the ovary of non-parthenocarpic

tomato was lower than that in *pat* (Olimpieri et al., 2007), *pat-2* (Fos et al., 2000), and *pat-3/pat-4* (Fos et al., 2001) parthenocarpic tomato. Several authors have reported that active GAs induced the development of seedless fruit (Bunger-Kibler and Bangerth, 1982; Sjut and Bangerth, 1982; Fos et al., 2000), and auxin increase the active GAs content in the fruit by unregulating genes encoding enzymes of GAs biosynthesis (Ross et al., 2000; Serrani et al., 2008). It is presumable that a high level of active or inactive GAs also inhibited seed development in parthenocarpic tomato.

This study showed that a high concentration of IAA inhibited the elongation of pollen tubes at the style base in *pat-2* parthenocarpic tomato, resulting in poor fertilization and failure of ovule development. As floral organs in *pat-2* parthenocarpic tomato were normally developed, seed development could be induced in *pat-2* parthenocarpic tomato by PCIB treatment. Parthenocarp is mainly used for crops where seedless fruit are desired by consumers (Varoquaux et al., 2000). In tomato, parthenocarp could be useful as an answer to global warming. However, the price of seed in parthenocarpic tomato was more expensive than that of non-parthenocarpic tomato because of seedless, and it restricted the distribution of parthenocarpic tomato. Therefore, it is absolutely essential to control the effect of PCIB on seed formation in other genotypes of tomato parthenocarpic such as *pat* and *pat-3/pat-4* genes. New or improved method by which seed formation in parthenocarpic tomato was induced more than by that in this study was needed.

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