Density effects on late flowering mutants of *Arabidopsis thaliana* under continuous light

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**Abstract**  In general, plant growth is inhibited under high-density conditions, while it is promoted under low-density conditions. This is known as the "density effect". Growing plants at high densities is often associated with an accelerated flowering time. Three major pathways [the long day (LD), gibberellic acid (GA), and autonomous/vernalization pathways] are known to play important roles in the control of flowering time. Circadian clock genes, namely, *LHY*, *CCA1*, *GI*, and *ELF3*, regulate the LD pathway. GAI and FCA control flowering via GA and autonomous pathways, respectively. The density effect on plant size is caused by specific factors such as the amount of nutrition obtained from the soil and touch frequency among plants. However, the molecular mechanism underlying the acceleration of flowering time due to density effects remains unclear. Here, we show the density effects on three Brassicaceae plants, namely, *Brassica rapa* var. *nipposinica*, *B. napus*, and *B. chinensis* f. *honsaitai*. They showed shorter stems and leaves when grown at high densities on soil under continuous light (LL). Shorter stems and leaves, as well as accelerated flowering times, were observed when a model plant, *Arabidopsis thaliana*, was grown under the same conditions. Unexpectedly, *ethylene insensitive 2* (*ein2*) showed no differences in density effects in our experiments. The acceleration of flowering at higher densities was largely suppressed by *gai*, but not by *gi*, *lhy;cca1*, or *fca*. These results suggest that the promotion of flowering (as a density effect) is likely dependent on the GA pathway, but not the LD or autonomous pathways.

**Key words:**  *Arabidopsis*, density effects, flowering time, gibberellic acid, law of constant final yield.

Environmental factors that affect plant growth include rain, wind, sunlight, and nutrition in soil. Plant density also affects plant growth. When plants are grown at high densities, the amount of nutrition from the soil decreases, and touch frequency among plants and the amount of plant hormones and growth substances that are released from plants increases (Harper 1977). Generally, plants grow smaller under high-density conditions and larger under low-density conditions (with proper spacing between plants) in what is called a "density effect" (Pacala and Weiner 1991). The stem or hypocotyl lengths, size of leaves and flowering time are often used as indicators of plant growth to investigate density effects (Akihama 1968; Jennings and de Jesus 1968; Levin and Wilson 1978).

Density effects have been reported in plants, echinoderms, protozoans, bacteria, isopoda, and insects (Fishman 1997). Competition, cooperation, and environmental factors such as light, temperature, and food determine the population density (Scrimshaw 1966). Grasshoppers grown in low-density populations live alone, but those grown in high-density populations possess better wings and live in groups. Grasshoppers grown in high-density populations often jump and move to other locations because obtaining sufficient food is difficult in the high population area. This behavior can cause locust plague and damage to crops. In this way, population density affects body weight, behavior, and oviposition of individuals, but in some species, it also affects morphology, color, and physiology (Scrimshaw 1966).

Plants under high-density conditions cannot change locations to locate nutrition, so the density effect mechanism differs. In general, plant weight under low-density conditions increases compared to high-density conditions because of the amount of available resources.
Density effects on late flowering mutants of Arabidopsis (Willey and Heath 1969). The yield is approximately fixed regardless of density, which is called the law of constant final yield (Kira et al. 1953; Pacala and Weiner 1991; Pearl and Parker 1922).

Ethylene is a simple gaseous plant hormone that affects numerous physiological processes in the growth and development of higher plants (Kendrick and Chang 2008). Plants release ethylene in response to environmental stresses such as being touched frequently, oxygen deficiency, wounding, and pathogen invasion. Increased ethylene levels can affect plant developmental processes such as seed germination and seedling growth, leaf abscission, organ senescence, and fruit ripening (Kendrick and Chang 2008). Many ethylene-related mutants of Arabidopsis thaliana (Arabidopsis) have been identified. For example, ethylene-insensitive mutants such as ethylene responsive 1 (etr1; Bleecker et al. 1988) and ethylene insensitive 2 (ein2; Guzmán and Ecker 1990) do not respond properly to ethylene. constitutive triple response 1 (ctr1) causes constitutive ethylene response and dwarf phenotype in Arabidopsis (Kieber et al. 1993). Therefore, ethylene has been suggested to play an important role in promoting the density effects on plant growth.

Acceleration of flowering time is known to be caused by a density effect (Akihama 1968). A transition from the vegetative phase to the reproductive phase is called flowering, which is controlled by at least three distinct pathways in Arabidopsis; the long-day (LD)/photoperiod pathway, the gibberellic acid (GA) pathway, and the autonomous/vernalization pathway. The LD/photoperiod pathway is mainly affected by the circadian clock (Mizoguchi et al. 2002, 2005). In Arabidopsis, clock proteins such as LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), and EARLY FLOWERING 3 (ELF3) play key roles in maintaining circadian rhythms (Alabadi et al. 2001, 2002; Mizoguchi et al. 2002). Two genes, GIGANTEA (GI) and CONSTANS (CO), promote flowering through the LD/photoperiodic pathway, while GIBBERELLIC ACID INSENSITIVE (GAI) and FLOWERING CONTROL LOCUS A (FCA) are floral activator genes of the GA and autonomous/vernalization pathways, respectively. FLOWERING LOCUS T (FT) is a floral hormone, florigen, which acts on downstream targets common to all three flowering pathways (Kardailsky et al. 1999; Kobayashi et al. 1999; Corbesier et al. 2007; Tamaki et al. 2007; Matsoukas et al. 2012; Tsuji et al. 2013). The genetic pathway GI-CO-FT plays a central role in the photoperiod-dependent acceleration of flowering in Arabidopsis (Mizoguchi et al. 2005).

For example, under both short-day (SD) and LD conditions, mutations in the circadian clock genes LHY and CCA1 (lhy;cca1) accelerate flowering (Mizoguchi et al. 2002, 2005), but delay flowering under continuous light (LL; Fujiwara et al. 2008; Yoshida et al. 2009). In contrast, elf3 flowers earlier than the wild-type (WT) under SD, LD, and LL (Yoshida et al. 2009; Zagotta et al. 1996).

To establish experimental conditions to investigate the density effects on plants, three Brassicaceae plants, namely, B. rapa var. nipposinica, B. napus, and B. chinensis f. honsaitai were used (Figure 1). Plant seeds at densities of 1, 4, 9, 16, 25, and 36 per pot were sown...
and plants were grown for 20 days under LL. At lower densities, the number of leaves of *B. rapa* var. *nipposinica* (Figure 1A, D), *B. napus* (Figure 1B, E), and *B. chinensis* f. *honsaitai* (Figure 1C, F) increased. For example, the average numbers of leaves of *B. rapa* var. *nipposinica* at densities of 1 and 36 were 4.5 and 2, respectively. In addition, as the density decreased, the leaf size increased (Figure 1A–C).

We next confirmed the density effect on Arabidopsis under our laboratory conditions. Ler WT seeds at densities of 5, 10, 20, 40, and 60 per pot were sown, and plants were grown for 35 days under continuous light conditions (LL). As the density decreased, the stem length and number of siliques increased (Figure 2A–C). For example, the average stem length at densities of 5 and 60 were 26.5 cm and 7.1 cm, respectively (Figure 2B). The average number of siliques at densities of 5 and 60 were 9.2 and 4.2, respectively (Figure 2C).

Next, the density effect on the rosette radius of Arabidopsis was examined under the same conditions. WT (Ler) seeds at densities of 5, 10, 20, 40, and 60 per pot were sown, and plants were grown for 1, 2, 3, and 4 weeks under LL (Figure 2D). No significant difference in rosette radius was observed at densities of 5–60 by 2 weeks, but after this time, the differences in size became significant between densities of 5–10 and 20–60. As the density decreased, the rosette radius increased with similar patterns as changes in stem lengths and the numbers of siliques. These results indicated that plant densities have significant effects on the stem length, number of siliques, and rosette radius in Arabidopsis WT (Ler) under LL.

These results on Arabidopsis WT (Ler), as well as *B. rapa* var. *nipposinica*, *B. napus*, and *B. chinensis* f. *honsaitai*, indicated that the laboratory conditions used in this study were appropriate to investigate the molecular mechanisms underlying density effects on plants.

To investigate the possible roles of ethylene on the density effects on flowering time, osette radius and stem
Density effects on late flowering mutants of Arabidopsis

length of Arabidopsis, WT (Col) seeds and ethylene-insensitive 2 (ein2) mutants were sown at densities of 5, 10, 20, 40, and 60 per pot, and plants were grown under LL (Figure 3). As the density increased, flowering time (Figure 3A), rosette radius (Figure 3C) and stem length (Figure 3D) of the WT (Col) plants decreased. ein2 did not significantly suppress the decrease in flowering time, rosette radius and stem length (Figure 3B–D), indicating that ethylene did not play a major role in the density effects on Arabidopsis.

Although accelerated flowering time is known to be a density effect (Akihama 1968), the molecular mechanism underlying this characteristic is not fully understood. In plants, three major genetic pathways, namely, the LD, GA, and autonomous/vernalization pathways, regulate flowering time (Koornneef et al. 1991). To investigate the roles of key regulator genes of the density effect in these Arabidopsis flowering pathways, four late and one early flowering mutants were examined (Figure 4). The Arabidopsis mutants, gi-6 (Ler; Koornneef et al. 1991), lhy-12;cca1–101 (Ler; Fujiwara et al. 2008), fca-1 (Ler; Koornneef et al. 1991), gai (Ler; Koornneef et al. 1991) and elf3–101 (Ler; Yoshida et al. 2009) were used in this work. Seeds of the WT (Ler), four late flowering mutants (gi, lhy;cca1, gai, and fca), and an early flowering mutant (elf3) were sown at densities of 5, 10, 20, 40, and 60 per pot, and plants were grown under LL. As the density increased, the total leaf number of Ler WT plants decreased (Figure 4A). The late flowering phenotypes of gi, lhy;cca1, and fca were largely suppressed as the...
density increased (Figure 4B–D). In contrast, the delay in flowering of gai was only slightly suppressed at high densities (Figure 4E). These results suggested that acceleration of flowering time at higher densities is dependent on the GA pathway and independent of the LD and autonomous pathways.

Acceleration of flowering at higher densities was not significantly affected by gi (Figure 4B). However, the early flowering phenotype of elf3 was largely suppressed by gi (Figure 4F, G), suggesting that the acceleration of flowering at increased densities was not dependent on ELF3 activity. Although the accelerated flowering time of the early flowering mutant elf3 at higher densities was only observed in 1.6 leaves, the difference was statistically significant compared to the WT (Figure 4F), suggesting that elf3 is sensitive to the high-density. These data

Figure 4. Density effect on flowering time of early and late flowering mutants of Arabidopsis. (A) Wild type (WT, Ler), (B) gi, (C) lhy;cca1, (D) fca, (E) gai, (F) elf3, and (G) elf3;gi. Seeds were imbibed and cold-treated at 4°C for 3 days in darkness before germination under light. Seeds (60, 40, 20, 10, and 5 per pot) were sown and plants were grown in pots (L=2 cm, D=2 cm, H=2 cm) supplemented with soil (Jiffy Mix, Sakata) in controlled-environment rooms at 22°C under continuous light (LL) with a photon flux density of ∼40 µmol m⁻² s⁻¹. Flowering time was scored by counting the number of rosette and cauline leaves on the main stem after bolting. Data are presented as means±SE (n=10). Each experiment was performed at least twice with similar results.
Density effects on late flowering mutants of Arabidopsis supported our hypothesis that an increased density and elf3 promoted flowering through two distinct pathways.

Flowering time, rosette radius, and stem length were affected by higher densities. To determine whether the GA pathway plays a role in the control of rosette radius and stem length, seeds of gai and other controls were sown and plants were grown in pots (L=2 cm, D=2 cm, H=2 cm) supplemented with soil (Jiffy Mix, Sakata) in controlled-environment rooms at 22°C under continuous light (LL) with a photon flux density of ~40 µmol m⁻² s⁻¹. Rosette radius of plants was measured 3 weeks after sowing seeds. Data are presented as means ± SE (n=10). Each experiment was performed at least twice with similar results.

Figure 5. Density effect on rosette radius of early and late flowering mutants of Arabidopsis. (A) Wild type (WT, Ler), (B) gi, (C) lhy;cca1, (D) fca, (E) gai, (F) elf3, and (G) elf3;gi. Seeds were imbibed and cold-treated at 4°C for 3 days in darkness before germination under light. Seeds (60, 40, 20, 10, and 5 per pot) were sown and plants were grown in pots (L=2 cm, D=2 cm, H=2 cm) supplemented with soil (Jiffy Mix, Sakata) in controlled-environment rooms at 22°C under continuous light (LL) with a photon flux density of ~40 µmol m⁻² s⁻¹. Rosette radius of plants was measured 3 weeks after sowing seeds. Data are presented as means ± SE (n=10). Each experiment was performed at least twice with similar results.
stem length at higher densities, this process should be suppressed by gai. However, stem length of gai at a density of 5 was significantly reduced compared to the WT (Figure 6A, E, F). Reduction of stem length in gai still occurred at densities of 60 (Figure 6E, F), suggesting that GA did not play an important role in high-density-dependent shortening of stem length. Loss of function of GI, LHY, CCA1, and FCA did not significantly affect the reduction of stem length at higher densities (Figure 6).

As shown in Figures 1–6, our results in the Arabidopsis WT (Ler and Col) and Brassicaceae (B. napus, B. rapa var. nipposinica, B. chinensis f. honsaitai) plants indicated that plants grow smaller under high-density conditions and larger under low-density conditions (with proper spacing between plants). This is known as the "law of constant final yield" (Pacala and Weiner 1991).

Ethylene is thought to play an important role in
density effects. Therefore, we expected that ethylene-
insensitive mutants (etr1, ein2, ein3, and ein5; Bleecker et al. 1988; Guzmán and Ecker 1990) would grow larger
than the WT at high densities. However, ein2 and the WT showed no significant difference in rosette radius,
stem length, or flowering time (Figure 3), indicating that
ethylene does not play a major role in density effects
under our experimental conditions. However, a detailed
analysis of other ethylene-related mutants such as
etr1, ein3, ctr1, eto1, eto2, and eto3 (Bleecker et al. 1988;
Guzmán and Ecker 1990) is required. Based on these
results, unidentified factors other than ethylene may play
a role in the density effects in plants.

We proposed that the accelerated flowering time
could be used to investigate density effects. Regulation
of flowering time has been well studied because it is
stable and easy to investigate. Many genes that control
flowering time have been identified in model plants such
as Arabidopsis (Song et al. 2013), Oryza sativa (Tsui et
al. 2011), and Pisum sativa (Weller et al. 2009).

At high densities, the Arabidopsis WT (Ler and Col)
flowered earlier than at low densities. The acceleration
of flowering time at high densities is not likely due to
shade avoidance alone. Although the acceleration of
flowering time at higher densities was not affected by
gi and lhy;cca1, the early flowering phenotype of phyB
mutants was partly suppressed by a mutation in the
CO gene, which is a downstream factor of GI, LHY,
and CCA1 in the LD pathway (Mizoguchi et al. 2005;
Putterill et al. 1995; Reed et al. 1993). In contrast, gai
appeared less-sensitive to density effects, suggesting
that the acceleration of flowering at high densities may
require key components of the GA pathway. Whether
other mutations in the biosynthesis and signaling of GA
affect the acceleration of flowering time at high densities
remains unknown.

Florigen is a plant hormone that accelerates flowering
time (Matsoukas et al. 2012; Tsui et al. 2013). FT
encodes florigen, and many genes in the three major
flowering pathways positively and negatively affect the
gene expression of FT (Corbesier et al. 2007; Kardailsky
et al. 1999; Kobayashi et al. 1999; Tamaki et al. 2007).
Expression of the FT gene is likely increased in plants
grown at high densities. FT, as well as SUPPRESSOR
OF OVEREXPRESSION OF CO 1 (SOC1) and LEAFY
(LFY), are known to be floral integrators (Komeda 2004).
Expression of these three genes is increased by GA
(Mutasa-Göttgens and Hedden 2009). Detailed analysis
on the expression of the floral integrator and regulator
genes would increase our understanding of the molecular
mechanism underlying density effects.

Although GA appears to play a positive role in the
high-density-dependent acceleration of flowering, GA is
unlikely to have a major role in the control of stem length
and rosette radius at high densities (Figures 5 and 6).

Regulators of flowering time such as GI, LHY, CCA1, and
FCA are also unlikely to be involved in these processes
(Figures 5 and 6).

Identification of key steps in the biosynthesis or
signaling of GA for acceleration of flowering at high
densities is an important challenge. Thus, whether GA-
insensitive or -deficient mutants in other plant species
show similar phenotypes as Arabidopsis gai should be
explored. Genetic screening of Arabidopsis mutants in
which stem length, rosette radius, or flowering time are
not affected by high densities is important to understand
the molecular mechanisms underlying the law of
constant final yield.

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