Live-imaging evaluation of the efficacy of elevated CO₂ concentration in a closed cultivation system for the improvement of bioproduction in tomato fruits

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Abstract To maximize fruit yield of tomatoes cultivated in a controlled, closed system such as a greenhouse or a plant factory at a limited cost, it is important to raise the translocation rate of fixed carbon to fruits by tuning the cultivation conditions. Elevation of atmospheric CO_2 concentration is a good candidate; however, it is technically difficult to evaluate the effect on fruit growth by comparing different individuals in different CO_2 conditions because of large inter-individual variations. In this study, we employed a positron-emitting tracer imaging system (PETIS), which is a live-imaging technology for plant studies, and a short-lived radioisotope ¹¹C to quantitatively analyze immediate responses of carbon fixation and translocation in tomatoes in elevated CO_2 conditions. We also developed a closed cultivation system to feed a test plant with CO_2 at concentrations of 400, 1,500 and 3,000 ppm and a pulse of ¹¹CO₂. As a result, we obtained serial images of ¹¹C fixation by leaves and subsequent translocation into fruits saturated at 1,500 ppm on average. The translocation rate had larger inter-individual variation and showed less consistent responses to external CO_2 conditions compared with carbon fixation. Our experimental system was demonstrated to be a valuable tool for the optimization of closed cultivation systems because it can trace the responses of carbon translocation in each individual, which are otherwise usually masked by inter-individual variation.

Key words: CO₂ elevation, live-imaging, PETIS, tomato, translocation.

Tomato (Solanum lycopersicum L.) is one of the most popular crop plants for cultivation in a greenhouse. In the context of productivity, it should be stressed that the purpose is to obtain the best yield of the fruits and not the largest biomass in other parts (e.g. leaves, stems and roots). In other words, the source-sink balance of carbon nutrition in the plant body should be a key aspect for optimizing the cultivation systems. Mature leaves (typical "source") produce photoassimilates and maturing fruits (typical "sink") receive them, but the total resources, such as energy input to the leaves and the space for cultivation, are always economically limited in commercial production. Kato et al. (2011) examined the effects of photosynthetic photon flux (PPF) in their plant factory on the yield of miraculin in transgenic tomato fruits, and showed that the best cost performance was attained at PPF 300 (μ mol m⁻² s⁻¹) when the cost of electricity for the light supply was taken into consideration.

Elevation of CO₂ concentration is employed widely in the greenhouse cultivation of tomatoes in agriculture to increase the yield, and seems to have a potential to change the source-sink balance. Tripp et al. (1991) demonstrated that fruit yield of eight genotypes of tomato was enhanced by 7-34% by feeding CO₂ to 1,000 ppm in a greenhouse, and reported that the enhancement was directly correlated with decrease in root fresh weight, but not with increase in foliar photosynthetic activity. Duan et al. (2014) employed ¹⁴CO₂ and 18-day-old Arabidopsis thaliana seedlings to demonstrate that cultivation under increased CO₂ (780 ppm), in comparison with ambient CO₂ (390 ppm), conferred a higher capacity for translocation of photoassimilates from source leaves to sink organs probably by up-regulation of genes related to photosynthesis and sucrose transport, and of plasmodesmal biogenesis. Therefore, elevation of CO₂

Abbreviations: LED, light-emitting diode; PETIS, positron-emitting tracer imaging system; ROI, region of interest. This article can be found at http://www.jspcmb.jp/

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may also affect the source–sink balance in tomato in closed cultivation systems. However, in contrast to *A*. *thaliana* seedlings, mature tomato plants with developing fruits have large inter-individual variation in the numbers, positions and sizes of source and sink organs, and thus it is technically difficult to examine the effect of CO_2 elevation on source–sink balance by comparing different individuals in different CO_2 concentrations.

To solve this technical problem, we utilized a positron-emitting tracer imaging system (PETIS), which is a live-imaging technology using radiotracers specially designed for plant studies. PETIS provides serial time-course images (i.e. movies) of the twodimensional distribution of a radiotracer within intact plants without contact, and has been employed for over a decade in studies of absorption, fixation, transport and accumulation of various elements such as carbon (Kawachi et al. 2011), nitrogen (reviewed in Fujimaki et al. 2010a) and cadmium (Fujimaki et al. 2010b). In particular with carbon, it is a remarkable advantage of PETIS that repeated experiments are possible because the measurement is fully noninvasive and the radioisotope, ¹¹C, used as the tracer decays sufficiently within a few hours after use (half-life: 20.39 min). The instant effects of external conditions on photoassimilate translocation have been examined using ¹¹CO₂ and PETIS by comparing results from the same individuals in different conditions. A promoting effect of CO₂ elevation was quantitatively estimated on the flow speed of photoassimilates from a source leaf through the stem in broad bean plants (Matsuhashi et al. 2005). It was also reported that salt treatment of tomato roots with 150 mM NaCl halved the flow speed of photoassimilates in the stem within 2h, although the photosynthetic activity in the source leaves was not affected until 5 h (Suwa et al. 2008).

In this study, we employed ${}^{11}CO_2$ and PETIS to evaluate the efficacy of elevated CO_2 concentration on the bioproductivity of tomato fruits in a closed cultivation system. The rates of carbon fixation and translocation to fruits were analyzed quantitatively in same individual tomato plants when exposed to elevated CO_2 levels.

Materials and methods

Plant materials

Seeds of tomato (*Solanum lycopersicum* L. cv. 'Micro-Tom' (Scott and Harbaugh 1989)) were surface sterilized with 5 gl^{-1} sodium hypochlorite solution, and sown on wet filter paper. Seedlings were transplanted into rockwool pots 1 week after sowing and then into polyethylene pots (90 mm in diameter) containing common culture soil with sufficient nutrition, and grown for 1 week at 25°C with a light intensity of 100–120 μ mol m⁻²s⁻¹ (measured using an MQ-200, Apogee Instruments, Inc., Logan, USA) with a 16 h/8 h light/dark photoperiod and relative humidity of 65%.

All the lateral buds were removed, and inflorescences were excised except the first and second ones to simplify the plant structure. These excisions were completed before the first blooming of a flower in the first inflorescence. Six plants at 56 days after sowing with the following conditions were selected and subjected to the experiments: 10–11 true leaves, two inflorescences without any branches, more than three fruits per inflorescence, and fruits at immature or mature green stages only (Yin et al. 2010). The test plants were grown in continuous light for 3 days before the experiment to override the circadian rhythm, which may obscure the results.

Gas-conditioning system

A clear acrylic air-tight chamber was developed to feed whole body of the test plant with conditioned air and ${}^{11}CO_2$ tracer (Figure 1A). The chamber was 224 mm in length, 144 mm in width and 291 mm in height, which was large enough to contain a whole Micro-Tom plant but small enough to set between the detector heads of the PETIS (Figure 1B). The chamber consisted of two components: a base plate and a cover



Figure 1. Experimental system. A, scheme of the gas-conditioning system for ${}^{11}CO_2$ feeding to test plants in controlled conditions. B, set-up of the chamber containing a test plant and the detector heads (indicated with stars) of the PETIS apparatus.

box with an airtight seal consisting of polyurethane rubber packing and screws to connect the components together to prevent gas leakage. The cover included two inlets for gas supply in the upper part and four exhaust outlets in the lower part, each connected to 4mm diameter tubes. Two small fans were also installed in the lower part to stir and quickly equilibrate the supplied gas. The sensor head of a multi-detector (C2D-H10; U-DOM Co., Ltd., Mito, Japan) was installed on the side of the chamber to monitor simultaneously the temperature, humidity, and CO₂ concentration in the chamber. Air at 25°C containing the set concentrations of CO_2 (400, 1,500 or 3,000 ppm) flowed into the chamber at rate of 41 min⁻¹ throughout the experiments. The concentration and flow rate were maintained using supply combinations from a compressed CO₂ gas cylinder, an air pump and a mass flow controller (5850i; ITW Japan LLC., Tokyo, Japan or FCC-3000-G2; KOFLOC Co., Ltd., Kyoto, Japan). The exhaust gas was passed into a vessel containing soda lime (Soda lime No.1; Wako Pure Chemical Industries, Ltd., Osaka, Japan) to completely collect the residual ¹¹CO₂.

PETIS measurement

The leaves of each test plant were gently bent down using a nylon net and strings to set them into the field of view of the PETIS and to prevent them from covering the fruits. The surface of the soil was covered with a plastic film or aluminum foil to prevent drying out of the soil surface. A wet paper towel was spread on the dish under the pot to maintain the humidity inside the chamber. The pot was placed on the center of the base plate and sealed in with the cover box, then the whole chamber was placed between the detector heads so that the fruits inside were located in the focal plane—the mid-plane of the surfaces of detector heads of the PETIS (Figure 1B).

Two opposing detector heads of the PETIS apparatus (modified type of PPIS-4800; Hamamatsu Photonics, Hamamatsu, Japan) installed in a plant growth cabinet were set 20 cm apart from one another (Figure 1B). The principle of image acquisition by PETIS is as follows (see Uchida et al. 2004 for details). A positron, the antiparticle of an electron, emitted from the tracer immediately undergoes annihilation by collision with an electron of an adjacent atom in the plant tissue. A pair of γ -rays are emitted in opposite directions from that point. The detector heads detect the pair of annihilation γ -rays at the same moment. Thus, the emission point is determined as the middle point of the two incident points. Repeated determinations of the emission points reconstruct one static image of the tracer distribution.

Continuous and constant light was provided for the test plants throughout the experiments at a photosynthetic photon flux of $120-160 \,\mu \text{mol m}^{-2} \text{s}^{-1}$ using LED lights (ISL-150×150-H2WW; CCS Inc., Kyoto, Japan).

Exactly 30 MBq of ¹¹CO₂ tracer was prepared (described in the next section) and loaded rapidly into the upstream tube of the gas-conditioning system to provide a pulse-feed of tracer to the test plant. At the same time, PETIS measurements were started. Ten seconds of image acquisition by PETIS provided one static image of ¹¹C-distribution, and in total 2.5 h of measurements provided 900 serial images. The same individual plant was subjected to a total of three successive measurements with freshly-prepared ¹¹CO₂ and different concentrations (400, 1,500 and 3,000 ppm) of CO₂. The intervals between the measurements were set as more than 15 min to confirm that the altered CO₂ concentrations in the chamber were stable. In theory, the residual activity of ¹¹C fed at the beginning of preceding measurement should have decayed to less than 0.4% of the original level when a new measurement was started. Three of the six test plants were examined in the order 400, 1,500 and 3,000 ppm, and the other three in the order 3,000, 1,500 and 400 ppm.

Production of ¹¹CO₂ tracer gas

The ¹¹CO₂ gas was produced via the nuclear reaction ¹⁴N(p, α)¹¹C by bombarding a pure nitrogen gas target with a proton beam at an energy of approximately 10 MeV from the AVF cyclotron of the Takasaki Ion Accelerators for Advanced Radiation Application (Japan Atomic Energy Agency). The resultant 40–60 MBq of ¹¹CO₂ (corresponding to approximately 0.12–0.18 pmol) was collected into the trap consisting of a stainless steel pipe cooled with liquid nitrogen and its radioactivity measured; it was then transferred to the gas-conditioning system. The correct timing to feed exactly 30 MBq of ¹¹CO₂ to the plant was calculated based on the measured radioactivity and the decay rate of ¹¹C (half-life: 20.39 min).

Data analyses

The image data were automatically corrected for the decay of ¹¹C and exported from the PETIS apparatus. Therefore, all the images and graphs shown represent the amount of total carbon, not only ¹¹C, which was originally fed together to the plant. NIH Image J 1.47v software (http://imagej.nih.gov/ij/) was used for image analyses. Regions of interest (ROIs) were selected manually from the image data (Figure 3B, Supplemental Figure 1A) and the time–activity curves, time-courses of decay-corrected radioactivity in the ROIs, were generated.

Results

Dynamic images of photoassimilate translocation

Carbon fixation and translocation in the whole bodies of six tomato plants at the fruit-developing stage were observed by PETIS with ¹¹CO₂ tracer successively with three different concentrations (400, 1,500 and 3,000 ppm) of total CO₂. The numbers and weights of fruits on the test plants are shown in Table 1. Representative PETIS images obtained from one individual are shown in Figure 2. It should be noted that all the data in this study were already corrected for the decay of ¹¹C and normalized as follows to quantitatively reflect the distribution and movement of total carbon originally fed to the plants with ¹¹CO₂ tracer. In general, when a radioisotope is

Table 1. Numbers, average weights and total weights of fruits on the test plants. All the fruits were at immature or mature green stages.

Individual	a	b	с	d	e	f
Number	7	11	5	7	8	6
Mean F. W. [g]	2.04	1.73	2.22	3.59	2.98	1.91
Total F. W. [g]	14.3	19.0	11.1	25.1	23.9	11.5



Figure 2. Imaging of carbon movement using PETIS. A, photograph of a test plant (bar: 10 cm). B, serial images obtained from the same individual plant with different concentrations of CO_2 . Signal intensity was corrected for the decay of ${}^{11}C$ and the ratio of ${}^{11}CO_2$ to total CO_2 in the fed gas. The image frames except the initial frame are displayed as the integration of 90 original frames (15 min). The frames after 90 min were omitted because of little change in the images.

fed as a tracer to a plant together with corresponding stable isotopes, the specific radioactivity (Bq mol⁻¹) is usually used for calculation of the absolute amount of the total (i.e. stable plus an ignorable level of radioactive) isotope. On the other hand, in this study, ¹¹CO₂ tracer was quickly injected into the air flow containing a set concentration of non-radioactive CO₂ through the gasconditioning system to make a pulse-feed of ¹¹CO₂ for the test plant. Therefore, the specific radioactivity in this case was variable, which makes it difficult to determine the absolute amount of carbon originally fed to the plant as CO₂ together with ¹¹CO₂ from the image data. However, the relative amount can be simply calculated by the following equation:

(Relative carbon amount $[count s^{-1} ppm]$)=(decaycorrected ¹¹C activity $[count s^{-1}]$)×(total CO₂ concentration in the fed air [ppm]).

In the initial frames, signals were saturated due to the introduction of ${}^{11}\text{CO}_2$ gas into the chamber, and then the gas was flushed out immediately. The fixed carbon gradually decreased in leaves and increased in fruits. Overall, the intensities of signals in the plant body appeared higher in experiments with higher CO₂ concentrations.

Time courses of relative carbon amounts in selected regions

The time course of relative carbon amount in a selected

ROI was estimated from the whole field of view of each image to evaluate the capacity of carbon fixation in the aerial part of the test plant. Figure 3A shows the results from a representative individual. The signal increased rapidly when the gas containing ¹¹CO₂ was introduced into the chamber and reached a peak within 1 min. Then, the signal dropped immediately due to flushing out of ¹¹CO₂ from the chamber and reached a plateau approximately 15 min after the start of the measurements. The relative carbon amount at this plateau was considered to represent the amount of fixed carbon in the plant, and was calculated as the average value from 15-20 min (denoted as C_{whole, initial}) for later analysis. Very similar curves were obtained in all the experiments with different CO₂ levels and different individuals, and C_{whole, initial} was estimated for each case.

Another time course of relative carbon amount was estimated from a ROI including the fruits in each image (Figure 3B) to evaluate the capacity of translocation to the fruit. Background activity was estimated from neighboring blank regions and subtracted from the time-activity curve from the fruits for background correction in advance (Supplemental Figure 1). The resultant curve is shown in Figure 3C. The relative carbon amount almost reached a plateau after 120 min, which was considered to represent the amount of translocated and accumulated carbon in the fruit. The average value at 120–150 min was calculated (denoted as $C_{\text{fruit, final}}$) for



Figure 3. Time courses of relative carbon amount in selected regions of interest (ROIs) from a representative individual. A, time courses of relative carbon amount in the whole field of view for the initial 30 min. The open circles, filled circles and open triangles indicate results obtained with 400, 1,500 and 3,000 ppm CO_2 , respectively. B, ROI selection on the image data (area enclosed by a dotted outline) for the estimation of time course of relative carbon amount in the fruits. C, time courses of relative carbon amount in fruits. Open circles, filled circles and open triangles indicate results obtained with 400, 1,500 and 3,000 ppm CO_2 , respectively. The data points before 15 min were omitted due to background correction.

later analysis. Very similar curves were obtained in all the experiments with different CO_2 levels and different individuals, and $C_{\text{fruit, final}}$ was estimated for each case.

Relative amounts of carbon fixation and translocation in different CO₂ concentrations

Figure 4A shows $C_{whole, initial}$ and $C_{fruit, final}$ for each individual plant in different CO₂ concentrations. It was shown that the amounts of fixed carbon ($C_{whole, initial}$) were similar on a logarithmic scale and were affected very consistently by different CO₂ concentrations among the test individuals. In contrast, the amount of carbon translocated into the fruits ($C_{fruit, final}$) showed larger variation and less consistent responses to elevated CO₂. Individuals a, b and c were subjected to a series of experiments in different CO_2 conditions in the order 400, 1,500 and 3,000 ppm, and individuals d, e and f in the order 3,000, 1,500 and 400 ppm. This was intended to examine a possible effect of acclimation by the test plants to the increasing or decreasing CO_2 levels; however, no consistent tendency in the two groups was found in Figure 4A, and thus no discrimination for the six individuals was made in the subsequent analyses.

The average changing rates of carbon fixation and translocation in elevated CO_2 conditions were estimated by normalizing the values of $C_{whole, initial}$ and $C_{fruit, final}$ with those in standard conditions at 400 ppm CO_2 , respectively (Figure 4B). It was shown that carbon fixation increased steadily by 1.5-fold at 1,500 ppm CO_2 and 2.1-fold at 3,000 ppm CO_2 on average. In contrast, translocation into the fruits was increased only by 1.28-fold at 1,500 ppm CO_2 and with a very large degree of variation, and there was no significant additional increase at 3,000 ppm CO_2 . As a consequence, the translocation rates of carbon, defined as ratios of translocated carbon to fixed carbon ($C_{fruit, final}/C_{whole, initial}$), showed a decreasing tendency in elevated CO_2 conditions (Figure 4C).

In order to explore a possible cause of the lower consistency of translocation rate among individuals than fixation capacity, the correlation of translocation rate with the number of or total weight of fruits was analyzed in each individual. As a result, there were positive correlations with the number of fruits to some extent (maximum $R^2=0.51$ at 400 ppm CO_2 , Figure 5A) but little correlation with the total weight of fruits (maximum $R^2=0.19$ at 400 ppm CO_2 , Figure 5B).

Discussion

In this study, we examined the immediate effects of elevated CO₂ concentrations in a closed cultivation chamber on the capacities of carbon fixation and carbon translocation into the fruits in the same individuals of tomato plants. A gas-conditioning system with an airtight chamber was developed that provided a constant flow of air with selected concentrations of CO₂, proper humidity and light, pulse-feeding of ¹¹CO₂, and liveimaging of the dynamic movement of carbon nutrition in combination with PETIS. As a result, it was successfully visualized over 150 min of serial images that ¹¹C was fixed by the leaves and other green organs and then translocated to the fruits with 400, 1,500 and 3,000 ppm CO_2 (Figure 2B). The series of image data in the same individual demonstrated quantitatively that carbon fixation was enhanced steadily by elevated CO₂ (Figure 4A, 4B). However, in contrast, carbon translocation into the fruit was not enhanced above 1,500 ppm and exhibited varied results at 3,000 ppm CO₂ (Figure 4A, 4B). The first clear conclusion from these results was that



Figure 4. Responses of carbon fixation and translocation to elevated CO_2 conditions. A, relative amounts of fixed carbon ($C_{whole, initial}$ open bars) and translocated carbon in the fruits ($C_{fruit, finab}$ filled bars) in different CO_2 concentrations in the respective test plants. a, b, c, d, e and f indicate individual plants (corresponding to those in Table 1). B. change in averages of relative amounts of fixed carbon ($C_{whole, initial}$) open circles) and translocated carbon in the fruits ($C_{fruit, finab}$ filled circles) in elevated CO_2 conditions. The data points indicate the means of measured concentrations of CO_2 in the chamber on the *x* axis and geometric means of ratios of the relative amounts compared with those at 400 ppm CO_2 on the *y* axis (*n*=6). Bars: standard deviations for the *x* axis, standard errors of the mean for the *y* axis. C, change in translocation rates ($C_{fruit, final}/C_{whole, initial}$) in different CO_2 concentrations in the respective test plants. a, b, c, d, e and f indicate individual plants (corresponding to those in Table 1) and Figure 4A).



Figure 5. A, correlation between the number of fruits and translocation rate. B, correlation between the total weight of fruits and translocation rate. In the both figures, open circles, filled circles and open triangles indicate results obtained with 400, 1,500 and 3,000 ppm CO_2 , respectively.

1,500 ppm CO_2 is the best among the three conditions tested for the best yield of carbon in fruits in Micro-Tom cultivation with our system. In a previous report with a greenhouse, the fruit yield of tomato was enhanced by 7–34% by feeding CO_2 to 1,000 ppm (Tripp et al. 1991). Our results for the enhancement of translocation of fixed carbon (Figure 4B) seemed to be in agreement with these results, although it should be stressed that there are many differences between long-term acclimation and shortterm response to elevated CO_2 (Makino and Mae 1999).

The decrease in translocation rate (Figure 4C) suggested three possible explanations: (1) larger amount of carbon stayed in the assimilated leaves or was translocated to other aerial part except the fruits, (2) carbon was translocated more preferentially to the roots instead of fruits, and (3) larger amount of carbon was emitted from the plant body by respiration. First, explanation (2) was unlikely because little signal was found in the root region in Figure 2B. It should be stressed that PETIS can easily visualize ¹¹C in the root system in a pot with soil (Kawachi et al. 2011) because the annihilation γ -rays have an energy (511 keV) high enough to penetrate the soil and pot. Second, explanation (3) was also unlikely because Figure 3A shows that there was little change after 20 min in the time courses

of relative carbon amount in the whole plant body, and the levels lasted until the end of measurements in most cases (data not shown). Therefore, explanation (1) was the most likely.

It was suggested that translocation rate has larger inter-individual variation and its response to surrounding CO₂ concentration was also less consistent compared with carbon fixation (Figure 4A, 4C), although the translocation rate is the most important index in the evaluation of source-sink balance. A possible cause was suggested from the difference in the number of fruits among the test plants (Figure 5A) but not difference in total weight (Figure 5B). Yin et al. (2010) suggested that carbon accumulation in fruits in Micro-Tom plants is active only in immature green and mature green stages but does not occur in the subsequent ripening stage. In this study, only fruits in these green stages were examined; therefore, it was likely that active accumulation took place in all the fruits. It was suggested by a mechanistic model that the carbon partitioning ratio between competing sink organs with different activity of carbon utilization (e.g. storage, growth or respiration) can be directly changed when the source supply is varied (Minchin and Lacointe 2005), which was also experimentally supported (Minchin and Thorpe 1996). Our results in Figure 5A can imply that the partitioning ratio between the fruits and other organs in the aerial part of Micro-Tom was partly dependent on the total number of the fruits under normal conditions, but the situation was immediately changed by increase of source supply under elevated CO₂ conditions.

From a methodological aspect, to the best of our knowledge this is the first experimental system employing a repeatable live-imaging technique to evaluate the effect of gas composition on the sourcesink balance for the optimization of closed cultivation systems. It was also demonstrated that, because the translocation rate had a large inter-individual variation, our system that can trace changes in translocation rate in the same individuals in various conditions offers advantages in estimation of bioproductivity in fruits. We consider that the conditions in plant factories, such as gas compositions, air flow, temperature, humidity, and light can be optimized by using an improved system with PETIS in the future.

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Supplemental Figure 1



Supplemental Figure 1 Background correction. A, selected regions of interest (ROIs) for fruits (orange outline) and blank regions (blue outline). B, original time–activity curves from fruits (open circles) and from blank regions (filled circles). It was assumed that the whole background curve from the fruits region is proportional to that from the blank regions, and the flat part at 15–30 min from the fruits region indicates the background level in this region because it is likely that ¹¹C-photoassimilates had not arrived at the fruits yet. Background correction was made by multiplying the blank-curve and subtracting it from the fruits-curve. It should be noted that this kind of background activity is often caused by blurred images of adjacent organs containing high radioactivity and thin tissues (e.g. leaves) due to escape of positrons without annihilation in the tissues.