Accelerating Soybean Breeding in a CO_2-Supplemented Growth Chamber

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Soybean (*Glycine max*) is the most important dicot crop worldwide, and is increasingly used as a model legume due to the wide availability of genomic soybean resources; however, the slow generation times of soybean plants are currently a major hindrance to research. Here, we demonstrate a method for accelerating soybean breeding in compact growth chambers, which greatly shortens the generation time of the plants and accelerates breeding and research projects. Our breeding method utilizes commonly used fluorescent lamps (220 μmol m^{-2} s^{-1} at the canopy level), a 14 h light (30°C)/10 h dark (25°C) cycle and carbon dioxide (CO_2) supplementation at >400 p.p.m. Using this approach, the generation time of the best-characterized elite Japanese soybean cultivar, Enrei, was shortened from 102–132 d reported in the field to just 70 d, thereby allowing up to 5 generations per year instead of the 1–2 generations currently possible in the field and/or greenhouse. The method also facilitates the highly efficient and controlled crossing of soybean plants. Our method uses CO_2 supplementation to promote the growth and yield of plants, appropriate light and temperature conditions to reduce the days to flowering, and the reaping and sowing of immature seeds to shorten the reproductive period greatly. Thus, the appropriate parameters enable acceleration of soybean breeding in the compact growth chambers commonly used for laboratory research. The parameters used in our method could therefore be optimized for other species, cultivars, accessions and experimental designs to facilitate rapid breeding in a wide range of crops.

Keywords: Accelerated generation • CO_2 supplementation • Crossing • *Glycine max* • Growth chamber.

Introduction

Soybean (*Glycine max* L. Merr.) originated in East Asia, including Japan, and is the most important dicot crop worldwide. The global production of soybean was the fourth highest of all crops in 2016, after maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (FAOSTAT database). Over the past two decades, worldwide soybean production and the area harvested have increased more rapidly than those of the other staple crops (FAOSTAT database), with soybean being increasingly used as a food oil, especially in emerging economies [United States Department of Agriculture (USDA) 2018]. Although the current major producers of soybean are the USA, Brazil and Argentina, soybean is an ancient food crop in China, Korea and Japan (Ohyama et al. 2013), cultivated for thousands of years, and numerous wild and cultivated soybean accessions originated in East Asia (Xu et al. 2002, Kaga et al. 2012). Soybean seeds are not only a major source of nutritious food for humans and livestock, but are also used for industrial materials, due to their high protein and oil contents (Yamada et al. 2012a).

The multifaceted importance of soybean has led to the development of a wide range of tools and genomic resources, which are easily accessible from public databases such as SoyBase (Grant et al. 2010), SoyKB (Joshi et al. 2012) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#) (Schmutz et al. 2010). Plant researchers can utilize a variety of robust molecular tools, including transformation (Hinchee et al. 1988, McCabe et al. 1988, Finer and McMullen 1991), virus-induced gene silencing (Zhang and Ghabrial 2006, Yamagishi and Yoshikawa 2009, Ogata et al. 2017) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) genome editing (Cai et al. 2015, Jacobs et al. 2015, Li et al. 2015). Artificial and natural mutant resources have been developed, including ethyl methanesulfonate (EMS) mutant libraries (Cooper et al. 2008, Hoshino et al. 2014, Tsuda et al. 2015, Lakhssassi et al. 2017, Espina et al. 2018) and the collation of selected sets of germplasm into core collections of soybean accessions (Kaga et al. 2012). These tools and resources have established soybean as a model legume crop. However, many plant researchers believe that soybean needs more light than model plants, and that crossing genotypes is more laborious (Talukdar and Shivakumar 2012). Moreover, the slow generation times of soybean remain a major barrier to soybean studies.

Recently, Watson et al. (2018) developed a method for speed breeding long-day crops such as wheat and barley (*Hordeum vulgare*) using a prolonged photoperiod, which reduces the generation time and facilitates efficient breeding; however, the speed breeding methods have not been reported in short-day soybean plants. Here, we describe a new method for accelerating soybean breeding in widely used compact growth chambers, which greatly reduces its generation time and facilitates soybean breeding and research programs.
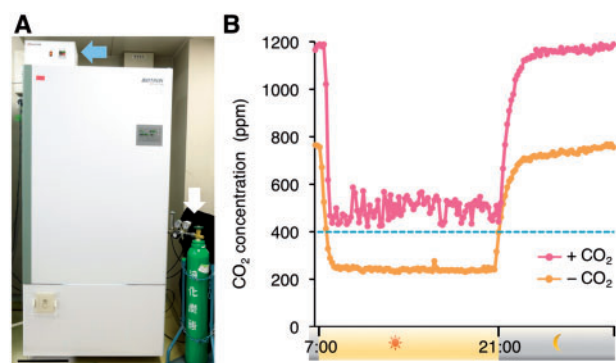
Fig. 1 Growth chamber CO_2 conditions over time. (A) A growth chamber used in this study. Blue and white arrows indicate the CO_2 regulator and the CO_2 cylinder, respectively. Scale bar = 30 cm. (B) The internal CO_2 concentrations within CO_2-supplemented (pink) and control (orange) growth chambers containing mature soybean plants. The data were collected every 10 min over a single day, 25 d after flower initiation in the soybean plants.

Results

CO_2 supplementation enhances the growth and yield of soybean plants in growth chambers

Here, we aimed to develop a method for accelerating soybean breeding using compact, environmentally controlled growth chambers (internal volumes of approximately 0.4 m^3), which are widely used in laboratory-based plant research (**Fig. 1A**). The chambers were fitted with commonly used fluorescent lamps, which provided a light intensity of around 220 μmol m^{-2} s^{-1} at the canopy level. As the appropriate cultivation area of each soybean cultivar is limited to relatively narrow latitudes (Watanabe et al. 2012), a single cultivar, the well-characterized elite Japanese soybean cultivar Enrei, was mainly used in this study (Supplementary Fig. S1). The genome sequence of Enrei has been published (Shimomura et al. 2015), and its genomic information is available in a public database, DaizuBase (Katayose et al. 2012). Moreover, a high-density mutant library, a high-density linkage map and chromosomal segment substitution lines have been developed for Enrei (Tsuda et al. 2015, Watanabe et al. 2018).

To evaluate soybean growth, 12 plants were grown in a single growth chamber, which was programmed for a photoperiod of 14 h of light at a temperature of 30°C, followed by 10 h of darkness at 25°C. These conditions are comparable with those of mid-summer in the plain regions of Japan (Japan Meteorological Agency, https://www.data.jma.go.jp/obd/stats/data/en/smp/index.html). Previous studies have shown that CO_2 concentrations inside growth chambers, which comprise a closed and limited space, were much lower than those of the ambient atmosphere (at approximately 400 p.p.m.) during the active growth phase of rice, and that CO_2 supplementation improved the growth and yields of rice grown in plant growth chambers (Ohnishi et al. 2011, Tanaka et al. 2016). To examine how exogenous CO_2 supplementation affects the CO_2 concentration inside growth chambers in the presence of soybean plants, the internal CO_2 concentrations were recorded.

During the light period, the internal CO_2 concentration ranged from 400 to 600 p.p.m. in the growth chambers supplemented with CO_2, whereas the CO_2 concentration in the unsupplemented growth chamber was consistently around 200 p.p.m. (**Fig. 1B**) at 25 d after flowering. During the dark period, the CO_2 concentration markedly increased to around 1,200 and 750 p.p.m. in the growth chambers with and without supplemental CO_2, respectively (**Fig. 1B**). The CO_2 concentrations in the growth chamber were changing depending on the soybean growth (Supplementary Fig. S2). These data suggest that the CO_2 concentration inside growth chambers is strongly affected by the photosynthesis and respiration of the soybean plants. In accordance with previous observations in rice (Ohnishi et al. 2011, Tanaka et al. 2016), our findings show that CO_2 concentrations within growth chambers are lower than the ambient CO_2 concentration during the day, which may retard plant growth.

We therefore investigated the effects of CO_2 supplementation on soybean growth in the growth chamber. Consistent with previous reports in rice (Ohnishi et al. 2011, Tanaka et al. 2016), we found that CO_2 supplementation enhanced the growth and development of soybean in the growth chamber in terms of total leaf area, plant height, total length of branches, dry leaf weight, dry stem weight and dry root weight (**Fig. 2;** Supplementary Fig. S3). We also evaluated the seed yields of the soybean plants, and found that the average seed numbers per plant were 53.4 ± 9.1 when supplemented with CO_2 and 26.6 ± 2.5 in the unsupplemented chamber (Supplementary Fig. S4A–F). In contrast, no significant difference was observed in the number of seeds per pod produced by plants grown in chambers with or without supplemental CO_2 (Supplementary Fig. S4G). We further evaluated the quality of the harvested seeds and found that the average seed weight was significantly increased by CO_2 supplementation (Supplementary Fig. S4E, H). These results therefore indicate that CO_2 supplementation enhances the growth and yield of soybean plants in the growth chamber.

Light and temperature regulate the days to flowering in soybean in growth chambers

Reducing the number of days to flowering is one of the most important factors for shortening generation times and accelerating breeding. Previous studies showed that CO_2 supplementation in growth chambers reduced the flowering time in rice (Ohnishi et al. 2011, Tanaka et al. 2016) and that low CO_2 concentrations delayed flowering in *Arabidopsis* (Li et al. 2014); therefore, we examined whether CO_2 supplementation affects the days to flowering in soybean plants grown in growth chambers. The number of days between sowing and flower initiation for plants grown in chambers with or without supplemental CO_2 was 25.5 ± 0.5 and 25.3 ± 0.5 d, respectively (**Fig. 3A**); thus, the differences in vegetative growth rates caused by CO_2 supplementation (**Fig. 2**) did not decrease the number of days to flowering. These results are consistent with the previous observations that flowering time is strictly controlled by temperature and photoperiod in soybean (Hadley et al. 1984, Cober et al. 2014). The data also indicate that the
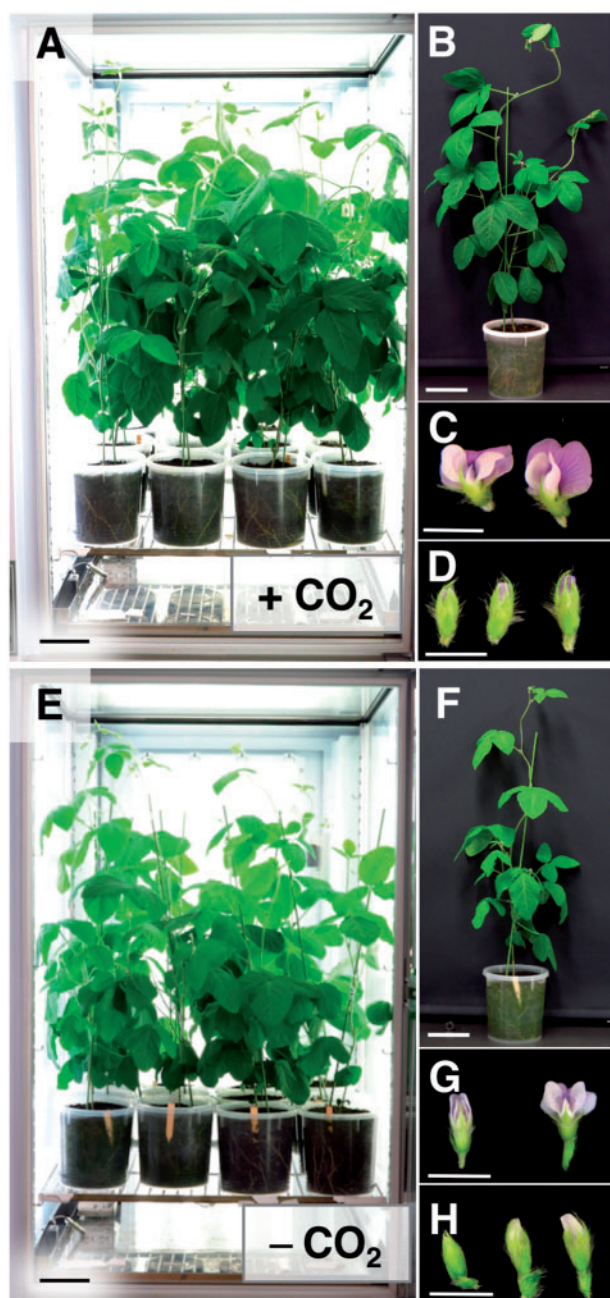
Fig. 2 CO_2 supplementation enhances soybean growth in growth chambers. Images show 27-day-old soybean plants and flowers grown in the compact growth chambers with (+; A–D) or without (−; E–H) supplemental CO_2. The growth chamber (0.4 m³) was programmed to run a 14 h light (30°C)/10 h dark (25°C) cycle. Twelve soybean plants in 2.0 liter pots were cultured in each growth chamber. Scale bars = 10 cm (A, B, E and F), 5 mm (C, D, G and H).

number of days to flowering of Enrei markedly decreased from 33–59 d reported in the field (Yamada et al. 2012b) to around 25 d in the temperature and light conditions used in this study. Likewise, the similar reductions in the days to flowering can be observed in the sequenced model US cultivar Williams 82 (Schmutz et al. 2010) (Supplementary Fig. S1) and the Brazilian cultivar BR16 (Supplementary Fig. S1), which are
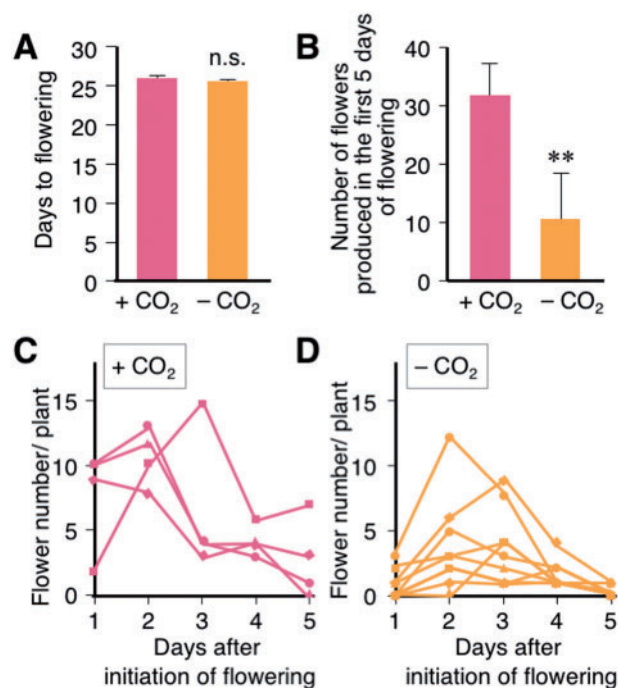

Fig. 3 CO_2 supplementation improves the quantity and quality of soybean flowers in the growth chamber. (A) Number of days to flower initiation after sowing soybean seeds in growth chambers with (+) or without (−) supplemental CO_2. Error bar = SD. $n = 8$. (B) Number of healthy flowers produced during the first 5 d of flowering in growth chambers with (+; $n = 4$) or without (−; $n = 8$) supplemental CO_2. Error bar = SD. (C, D) The number of healthy flowers produced each day for the first 5 d of flowering for plants grown with (C, $n = 4$) or without (D, $n = 8$) supplemental CO_2. **$P < 0.01$, n.s. no significant difference.

widely used in basic studies in Brazil (Rodrigues et al. 2015). The number of days between sowing and flower initiation decreased from >40–60 to 28.0 ± 0 d in Williams82 (Schon and Blevins 1990, Kong et al. 2018) (Supplementary Fig. S5), and also decreased from 33–44 to 30.4 ± 0.9 d in BR16 (Carpentieri-Pipolo et al. 2014). Thus, our results indicate that light and temperature conditions greatly contribute to reduce the days to flowering in soybean in growth chambers.

CO_2 supplementation improves the quantity and quality of soybean flowers, enabling higher crossing efficiency

The quantity and quality of flower buds in the female parent are important factors for efficient crossing. We analyzed the numbers of healthy flowers (**Fig. 2C**) produced by soybean plants grown in growth chambers with or without CO_2 supplementation. The average number of healthy flowers produced in the first 5 d of flowering was 32.0 ± 5.6 and 10.9 ± 7.9 for plants grown in growth chambers with and without supplemental CO_2, respectively (**Fig. 3B**). The peak numbers of healthy flowers were observed in the first 3 d of flowering (**Fig. 3C, D**). Abnormal flowers, which had immature or deformed corollas with insipid coloration, were often observed in the unsupplemented growth chamber (**Fig. 2G, H**), whereas abnormal
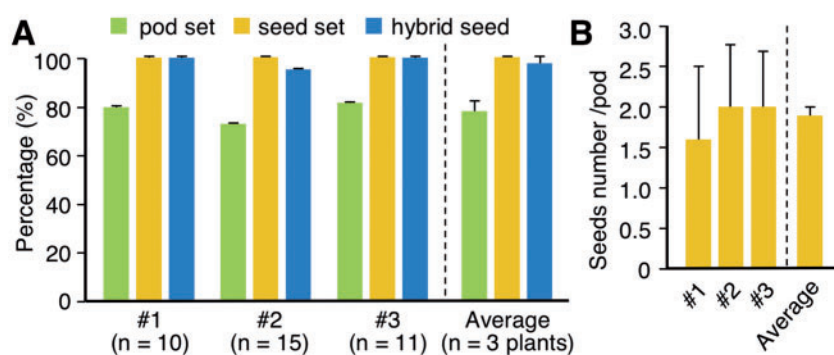
Fig. 4 The high crossing efficiencies in soybean plants grown in growth chambers supplemented with CO$_2$. (A) The percentage of pod set determined as the number of pods arising from the total number of crossings, and the percentages of seed set and hybrid seeds in the three individual female soybean parent plants. The total number of crossings in each plant was 10 in #1, 15 in #2 and 11 in #3. The average percentages from all three plants are shown on the right. Error bar = SD. (B) Seed numbers per pod in three individual female parent plants. The total numbers of pods in the three plants were 8 in #1, 10 in #2 and 9 in #3. The average seed numbers per pod from all three plants are shown on the right. Error bar = SD.

flowers were almost never seen during the first 5 d of flowering in plants grown in the CO$_2$-supplemented growth chamber (**Fig. 2C, D**).

Next, we examined whether healthy flowers could be obtained from plants grown in smaller pots (0.4 liter volume, 10 cm diameter) than those used throughout the rest of this study (2.0 liter volume, 15 cm diameter) in the CO$_2$-supplemented growth chamber. Many healthy flowers (25.3 ± 5.9 in the first 5 d of flowering) were observed in the vigorous soybean plants grown in these smaller pots (Supplementary Fig. S6). These observations suggest that the soybean plants grown in smaller pots produced almost as many healthy flowers as those plants grown in the larger pots, indicating that at least 30 plants could be grown for accelerating breeding in each growth chamber. These results show that CO$_2$ supplementation increases the amount of high-quality flowers produced by soybean plants in growth chambers, indicating that the flowers of soybean grown in the CO$_2$-supplemented growth chamber might be useful for performing effective crossings. Until now, conducting effective crossings out of season in artificial conditions was considered to be more challenging than doing so in the field in summer.

Therefore, to test the quality of the flowers produced by soybean plants grown in the CO$_2$-supplemented growth chambers, we investigated their crossing efficiency. Ten days after emasculating and pollinating the female flowers, 78.4 ± 4.5% of the crossed flowers had formed pods (**Fig. 4A**). The frequency of seed set within the pods was 100%, and the average number of seeds per pod was 1.9 ± 0.1 (**Fig. 4B**). The seeds were genotyped using a mutation as a marker of hybridization (Supplementary Fig. S7), revealing that 98.5 ± 2.6% of the harvested seeds were hybrids (**Fig. 4A**). The crossing efficiency was therefore 77.3 ± 6.4% for soybean plants grown in CO$_2$-supplemented growth chambers, which was much higher than that of field-grown soybean (Walker et al. 1979).
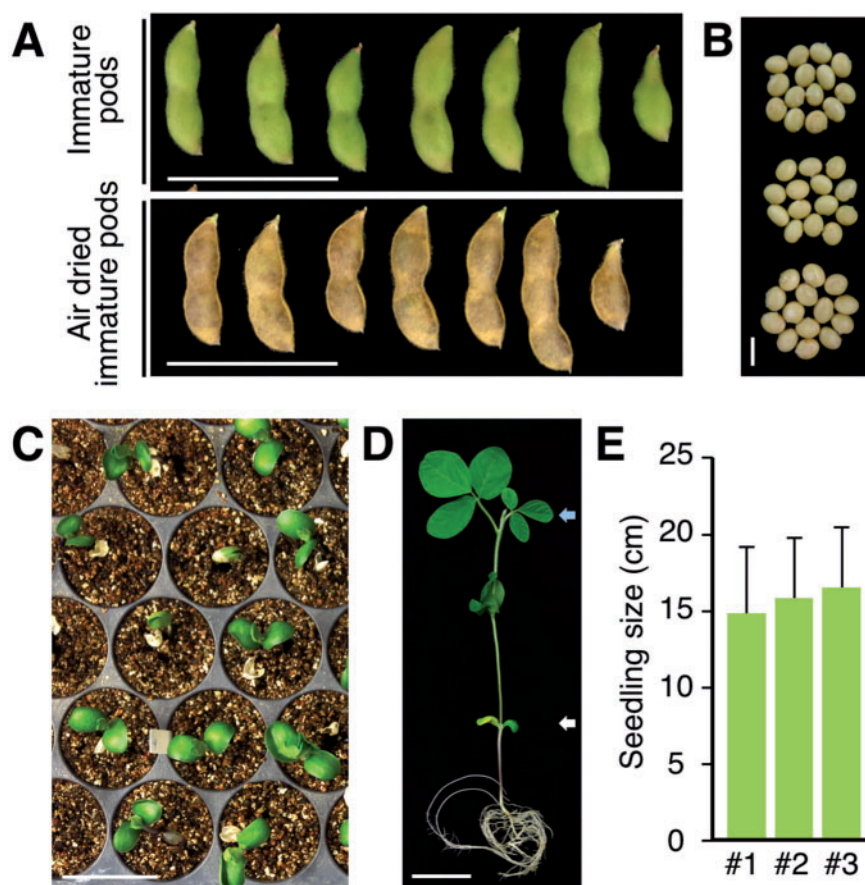
Air-dried immature soybean seeds display a high germination rate and normal seedling growth

The reproductive period of soybean comprises more than half its total life span (Roumet and Morin 1997). In the field in Japan,

Enrei was reported to take 65–92 d to complete seed maturation after flowering (Yamada et al. 2012b). To shorten this reproductive period, we examined whether the previously reported methods for germinating immature soybean seeds (Obendorf et al. 1980, Carandang et al. 2006) could be used to shorten the reproductive period of plants grown in CO$_2$-supplemented growth chambers. We harvested fully swollen immature pods at 37 d after flowering, as they started to change color from green to yellow (**Fig. 5A**). After air-drying the pods for 8 d, their color changed to light brown, and the seeds in the pods matured (**Fig. 5B**). Their germination rate at 7 d after sowing was 100% (**Fig. 5C**). By 15 d after germination, the seedlings had produced one or two trifoliate leaves (**Fig. 5D, E**). We have usually used the progeny grown from immature seeds as well as mature seeds for crossing. These data showed that the germination ratio and subsequent growth rate of these immature seeds were comparable with those of mature seeds, indicating that the harvesting and germination of immature seeds could be used to shorten further the generation time of soybean plants grown in CO$_2$-supplemented growth chambers.

Discussion

Here, we present a method for accelerating breeding of soybean in a compact growth chamber, which greatly reduces the generation time and facilitates rapid breeding and research projects. The recently reported speed breeding methods (Watson et al. 2018) have not been applied to short-day soybean plants; however, our method can reduce the generation time of the soybean cultivar Enrei, the best-characterized Japanese cultivar, to just 70 d (**Fig. 6**). This breeding approach therefore allows up to 5 generations per year, instead of the 1–2 generations currently possible under field and greenhouse conditions. Moreover, our method allows the highly efficient controlled crossing of soybean lines under laboratory conditions, enabling researchers and breeders to work consistently year-round, whatever the weather or season.

Fig. 5 Air-dried immature soybean seeds display high germination rates and normal seedling growth. (A) Immature soybean pods harvested 37 d after flower initiation, imaged both at the time of harvest and after 8 d of air-drying. Scale bars = 5 cm. (B) Soybean seeds removed from immature soybean pods air-dried for 8 d, taken from three independent plants. Scale bar = 1 cm. (C) Seven-day-old seedlings germinated from the air-dried immature seeds. The germination ratio of the air-dried immature seeds was 100% for seeds derived from three individual plants. *n* = 14 per plant. Scale bar = 5 cm. (D) A 15-day-old seedling derived from an air-dried immature seed grown on vermiculite. Scale bar = 5 cm. (E) The seedling size, determined as the stem length from the cotyledon [white arrowhead in (D)] to the top of the plant [blue arrowhead in (D)], of 15-day-old plants grown from immature seeds harvested from three individual plants. *n* = 14 per plant. Error bar = SD.
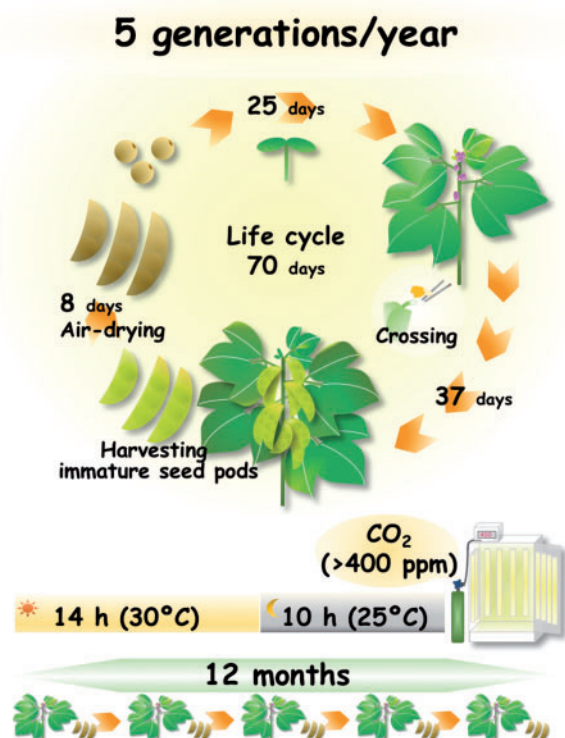
Our breeding method uses supplemental CO_2 in combination with appropriate light and temperature cycles to accelerate the growth and development of plants, and the reaping and sowing of immature seeds to greatly shorten the generation time further in the growth chamber. The compact chambers (0.4 m^3) used in this study are widely used for *Arabidopsis* research, thereby enabling plant researchers to shift their focus from this weedy model plant to soybean, a staple crop. Since the light intensity derived from general purpose fluorescent lamps was just 220 μmol m^{-2} s^{-1} at the canopy level in the growth chamber used in this study, the lighting costs (for both the light source and the electricity) would be lower than those described in recent speed breeding protocols, which use high-power light-emitting diodes (LEDs) providing light intensities of 340–650 μmol m^{-2} s^{-1} (Watson et al. 2018). Furthermore, our method uses a 14 h light period, which is much shorter than the 22 h period required by other speed breeding methods (Watson et al. 2018), contributing further cost savings.

So far, it has been difficult to obtain enough high-quality soybean flowers to perform effective crossings in artificial conditions, due to the less vigorous growth of plants compared with those in the field. We demonstrated that CO_2 supplementation enhances the quantity and quality of both vegetative and reproductive growth in soybean plants in the growth chamber (**Figs. 2, 3**; Supplementary Figs. S3, S4), and allows for the highly efficient controlled crossing of soybean. However, CO_2 supplementation is not directly involved in shortening the soybean life cycle. Instead, appropriate light and temperature cycles can be used to decrease the number of days to soybean flower initiation from 33–59 d (Yamada et al. 2012b) to 25 d, while the reaping and sowing of immature seeds greatly shortens the reproductive period from 65–92 d (Yamada et al. 2012b) to 45 d. Thus, the appropriate combination of supplemental CO_2, light and temperature conditions with the use of immature seeds would allow reduction of the generation time of Enrei from 102–132 d in the field (Yamada et al. 2012b) to just 70 d.

Considering the previous findings that Enrei is a weakly photosensitive cultivar (Ozawa et al. 2017) and that comparatively higher temperatures accelerate the flowering times in soybean plants (Hadley et al. 1984), our data suggest that the

of species, cultivars, accessions and experimental designs to facilitate cutting-edge breeding in a wide range of crops.

Materials and Methods

Plant materials and growth conditions

Soybean (*Glycine max* L. Merr.) cv. Enrei, Williams 82 and BR16 plants were grown in a 14 h light (30°C)/10 h dark (25°C) photoperiod for Enrei or in a 10 h light (30°C)/14 h dark (25°C) photoperiod for Williams 82 and BR16 in LH-410S growth chambers (Nippon Medical & Chemical Instruments) or LH-350S growth chambers equipped with the CO_2 regulator AMC-CO_2-1S (Nippon Medical & Chemical Instruments) and general purpose white light fluorescent tubes. Dedicated CO_2 regulators controlled the addition of CO_2 from external CO_2 cylinders to the growth chambers to maintain their internal CO_2 concentration at >400 p.p.m. The internal CO_2 concentrations were recorded every 10 min using a data logger (GL220-UM-801; GRAPHTEC). The light intensity (photosynthetic photon flux) in the growth chamber was around 220 μmol m^{-2} s^{-1} at the canopy level, while the relative humidity was not regulated and ranged from 50% to 80%.

The seeds were germinated on moistened vermiculite, and 10-day-old seedlings were transferred to individual plastic 2.0 liter pots filled with a mixture of Nippi soil (Nihon Hiryo) and Tsuchitaro soil (Sumitomo Forestry). Twelve 2.0 liter plant pots were placed in each growth chamber, and the positions of the plants were rearranged during watering. Additional fertilizer was not supplied after the seeds were sown.

Measurements of soybean growth

For the analyses of soybean growth in growth chambers with or without supplemental CO_2, 31-day-old soybean cv. Enrei plants were used. Total leaf area was measured using an automatic area meter (AAM-9; Hayashi Denko Co. Ltd.). For dry weight measurements, the parts of the plants were measured after being dried for 48 h at 60°C in an oven. The soybean plants were harvested at about 2.5 months after sowing, and then air-dried. Dried seeds were collected from the plants, which were used for the measurements of the seed weight.

Quantification of the days to flowering and the number of healthy flowers

The number of the days from sowing to the opening of the first flower was defined as the days to flower initiation. Newly opened healthy flowers (**Fig. 2C**) were counted each morning for the first 5 d after flower initiation. Aberrant flowers, with immature or deformed corollas and insipid coloration, were not counted.

Crossing soybean lines

Healthy flower buds of plants grown in the growth chamber, which were expected to open the next day and were swollen with a visible purple corolla through the calyx (**Fig. 2D**), were selected for crossing, and all the other flowers and buds at that node were removed using forceps. The calyx and corolla were detached, after which the immature anthers were carefully removed (emasculation). The pollen-containing anthers of male parents from newly opened flowers, which have a single nucleotide substitution in the Enrei genome background, were then gently attached to the stigma of the female emasculated flowers. Crossing was conducted for 3 d after the initiation of flowering using three independent female soybean parent plants in the growth chamber.

Evaluation of crossing efficiency

Almost 10 d after crossing, pods >1 cm in length were considered to be set, and the percentage of pod set was determined as the number of pods arising from the total number of crossings. The total number of seeds as a percentage of the total number of ovules in a pod at 30 d after crossing was also calculated to determine the seed set. To determine the number of hybrid seeds, a C-to-T mutation site derived from male parents was used as a marker. Total DNAs

Fig. 6 Schematic representation of our method for accelerating breeding in soybean (cv. Enrei) in a growth chamber supplemented with CO_2. The appropriate combination of a 14 h light (30°C)/10 h dark (25°C) photoperiod and >400 p.p.m. CO_2 concentration, with the use of immature seeds, reduces the generation time to 70 d, thereby achieving up to 5 generations per year in soybean. See main text for details.

temperature setting used in this study plays a more important role in the reduction in the days to flowering of Enrei. On the other hand, soybean plants are generally photosensitive, and thus there are variations in the days to flowering in soybean cultivars. However, although the cultivars used in this study, Enrei, Williams82 and BR16, greatly vary in cultivation areas (Alliprandini et al. 2009, Yamada et al. 2012b, Wolfgang and An 2017) (Supplementary Fig. S1), we showed that in these distinct cultivars, the days to flowering can be reduced with our method (Supplementary Fig. S5). Their growth habits are also different; Enrei and BR16 are determinate cultivars, whereas Williams 82 is an indeterminate cultivar (Oya et al. 2004, Tian et al. 2010, Yamada et al. 2012b) (Supplementary Fig. S1). As the latitude of cultivation areas and growth habits mainly depend on specific genotypes (Bernard 1972, Tian et al. 2010, Maldonado dos Santos et al. 2016, Langewisch et al. 2017), these findings suggest the possibility that our technique is of general application in a wide range of soybean cultivars. Given that each soybean cultivar can be cultivated only in limited latitudes (Watanabe et al. 2012), the photoperiod conditions among the parameters in our method needs to be adapted for each cultivar. Alongside other protocols for speed breeding and effective phenotyping (Kuroda and Ikenaga 2015, Watson et al. 2018), the parameters used in our method could be optimized for a variety

were extracted from 52 seeds harvested from the soybean plants 30 d after crossing, using a simple and rapid DNA extraction method (Edwards et al. 1991). A 207 bp sequence including the mutation site was amplified from the total DNAs using PCR and the primer pair 5′-CAAAACAAGCTTGACTCT TAAACTC-3′ and 5′-CGAAATAATACAAAACGGAGAATG-3′. The resulting PCR products were sequenced using the primer 5′-CAAAACAAGCTTGACTC TTAAACTC-3′. The sequences were aligned, and a heterozygous sequence at the mutation site indicated the hybrid seed derived from a successful cross.

Air-drying immature seed

Immature pods were harvested from 62-day-old soybean plants grown in the CO_2-supplemented growth chambers, 37 d after flower initiation. Fully swollen pods were collected as they began to change color from green to yellow (**Fig. 5A**). The harvested immature pods were air-dried for 8 d at room temperature at around 27°C. The immature pods were opened by hand after drying, and the seeds were sown on moistened vermiculite in the greenhouse at 27 ± 5°C and with a relative humidity of $50 \pm 15\%$. The germination ratio of the seeds was determined 7 d after sowing. The seedling growth, which was measured as the length of the stem from the cotyledon to the top of the plant, was measured in 15-day-old seedlings.

Supplementary Data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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