

A study on physiological mechanism of green stem disorder

in soybean (*Glycine max* (L.) Merr.)

- Analysis of inducing factors and evaluation of cultivar differences

with the light availability manipulation method -

2019

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Summary

Green stem disorder (GSD) in soybean retains green stems and leaves as the pods mature, thereby reducing the harvest efficiency. In order to elucidate the mechanism of GSD, the experimental treatment that promotes GSD needs to be established. The objective of this study is to develop and utilize a new method to promote GSD occurrences for studies of GSD mechanism.

Firstly, the effect of thinning on green stem disorder was analyzed. A cultivar 'Sachiyutaka' was grown at the experimental fields of NARO, Western Region Agricultural Research Center, Hiroshima, Japan, in the conventional crop management practices. Plant density was adjusted at the developmental growth stage R1 (the beginning of flowering) or at R5 (the beginning of seed filling), from dense (22.2 plants m⁻²) to sparse (5.56 plants m⁻²) by thinning. GSD occurrence was increased when plant density was changed, compared to the treatments that were maintained under either dense or sparse conditions. GSD was promoted more strongly when thinning was conducted at R5 than at R1 stage. And then a shading technique was employed at the field experiment to manipulate light-availability for the plants. Shading equipment surrounding plants, except for their upper-most leaves, was implemented to determine the association of shading and GSD. The results of the shade experiment revealed that GSD occurrence generally increased in treatments subjected to shade removal, compared to those that were shaded until R8 stage (full maturity) or never shaded since the time of sowing. GSD was strongly promoted by shade removal at R5 than at R1 stage. The shading results coincided with the results of the plant density experiment, indicating that an increase in light availability enhances source activity relative to sink at R5 stage, thereby promoting GSD occurrence

in soybean.

Secondly, the hypothesis that enhanced source ability by thinning or shade removal promoted GSD occurrences was verified. ‘Sachiyutaka’ soybean was grown in the field with the treatments of varied duration and timing of shade removal suggested that GSD severity became higher as duration of shade removal at R5 became longer and late timing of shade removal (28 days after R5) had no significant effect on neither GSD severity nor growth parameters related to source ability. The transcriptome analysis was conducted on the thinned and control plants. The result of transcriptome analysis of main stems at 14 days or 28 days after R5 using RNA-seq indicated that the thinned plants showed overrepresented upregulation of photosynthetic genes and upregulation of genes of vegetative storage proteins. The dry matter dynamics also was measured. The thinned plants showed increase in weight of vegetative parts. The increased vegetative biomass included dry weight of leaves per unit leaf area, which is considered associating increased physiological capacity of the leaves. These results suggested that GSD promoted by thinning and shade removal corresponded to excess source ability caused by increase in photosynthesis.

Thirdly, the thinning treatment was applied on breeding GSD insensitive cultivars. GSD insensitive cultivars is expected to be an effective countermeasure to GSD. However, it is difficult to stably detect cultivar differences in GSD under conventional field conditions, because the occurrences of GSD largely vary by location and year. Thus, the thinning treatment may help accurate phenotyping for occurrences of GSD in breeding. To verify this possibility, the thinning treatment was applied to the four cultivars, for which the GSD severity values had been evaluated in an independent study. As a result, the cultivar differences in GSD severity were generally comparable between the present and previous

studies. However, the difference was more evident when the thinning treatment was conducted, exhibiting the GSD score of 2.8 of ‘Hatsusayaka’ compared with the GSD score of 3.6 of ‘Sachiyutaka’. On the other hand, the scores of those cultivars were similar without the thinning treatment. A positive correlation between GSD severity and N concentration in the main stem could be seen but the increment of GSD score with unit increase of N concentration in the main stem differed between cultivars. Thus, although more cultivars need to be tested to prove, the thinning treatment could be useful as a phenotyping technique in the breeding of GSD-insensitive cultivars.

In these studies, it was indicated that light-availability manipulation methods could be the effective method to easily and stably generate GSD for study of GSD in both physiological mechanism and breeding. And I could indicate the results which supported the hypothesis that sink-source balance affects GSD occurrences by using the thinning treatment and the shade removal treatment.

Chapter 1

Introduction

1.1 Green stem disorder in soybean as a problem for farmers

Green stem disorder (GSD) in soybean is defined as a symptom in which stem and leaves stay green and retain some moisture even when pods normally matured (Harbach et al., 2016; Hobbs et al., 2006). GSD has also been termed delayed leaf senescence (Phillips et al., 1984), green-stem syndrome (Schwenk & Nickell, 1980), and inharmonious maturation (Furuya et al., 1988).

GSD is a problem for farmers because it negatively affects seed appearances and harvest efficiency. Seed appearances are impaired as seed surface is stained with the sap of moist stems and leaves when the plants are threshed in combine harvesters (Ogiwara, 2002). Harvest efficiency is greatly reduced compared to normally matured soybean plants because it is difficult to cut the moist stems of GSD soybeans by combine harvesters (Harbach et al., 2016; Hill et al., 2006).

Despite these problems, the mechanism of GSD occurrences has not been elucidated and

there is no effective countermeasure to GSD.

1.2 Hypothesis of the mechanism of GSD

The most measure hypothesis referred in many studies is that sink-source balance affects GSD occurrences. Hereafter, the sink means the plant organs where the assimilates such as photosynthate and nitrogen compounds accumulate, typically pod and seed organs. The source means the origin of the assimilates. And the sink-source balance is referred to as relative capacity of the organs for sink and source. Studies have suggested factors that promote GSD, such as excessively wet soil conditions during the reproductive period (Sato et al., 2007), drought at the flowering and pod set periods after excessively wet conditions during initial growth (Tsujimoto et al., 2006), high temperature during the reproductive period (Mochizuki et al., 2005), pest attacks at the pod set and filling stages (Boethel et al., 2000), and diseases occurrence (Takehara et al., 2016). It was also reported in many studies that depodding (cutting off pods artificially) at the pod set and filling stages delayed plant senescence including GSD (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982). The common feature behind of these treatments

is thought to be a decrease in the number of pods due to some type of stress during the reproductive phase. The pod number crucially limits the number of seeds, which plays a role as the primary sink during the seed filling period.

It was reported that total dry matter of vegetative organs increased in depodded plants (Heitholt & Egli, 1985). Wittenbach (1982) indicated that depodded plants showed increased leaf dry weight, starch content, and soluble protein content compared to the control plants. Depodding leads to the accumulation of vegetative storage proteins (VSPs) in leaves and stems (Ogiwara & Ishikura, 1994; Wittenbach, 1983a, 1983b). VSPs can accumulate and represent up to 45% of total leaf soluble protein (Mason & Mullet, 1990). VSP mRNA increased in depodded plants and mRNA levels correlated with VSP protein accumulation (Mason et al., 1988, Staswick, 1988, Staswick, 1989). Vegetative lipooxygenases, which were thought to function as a storage protein, also accumulates in leaves of depodded plants (Tranbarger et al., 1991) and expression of their mRNA also was induced by depodding (Bunker et al., 1995). Male-sterile soybeans, which could be a sort of genetically sink-limited plants, showed delayed leaf senescence (Burke et al., 1984; Brown & Hudson, 2017).

These studies have implied that GSD occurrence is related to a relative increase in source levels resulting from sink limitation (sink-source balance hypothesis). However, the

conclusive evidence of the sink-source balance hypothesis has not been shown due to methodological limitation discussed later and GSD has sometimes been observed without reduction in the number of pods (Mochizuki et al., 2005).

There is another hypothesis related to GSD which was called ‘the death hormone hypothesis’ (Engvild, 1989). In this hypothesis, some substance transported from fruits to vegetative organs promote senescence of vegetative parts.

It was reported that Bean pod mottle virus (BPMV) was the main cause of GSD (Schwenk & Nickell, 1980). However, Hobbs et al. (2006) indicated that there was no direct association between GSD occurrences and BPMV infection.

Hill et al. (2013) showed that fungicides application promoted GSD incidence although these results were not related to sink-source hypothesis.

In order to elucidate the underlying mechanism of GSD, it will be effective to find factors or experimental treatments that promote GSD, and then to analyze the effects of these treatments on physiological processes in soybean plant.

1.3 The possibility of breeding GSD insensitive cultivars

It has been reported in many studies that there are differences in GSD occurrences

between cultivars (Fujii et al., 2015; Furuya & Umezaki, 1993; Hill et al., 2006; Isobe et al., 2015; Matsumoto et al., 1986; Mochizuki et al., 2005; Pierce et al., 1984; Yamada et al., 2014).

There are some studies which showed factors to affect cultivar difference in GSD. Pierce et al. (1984) demonstrated that determinate genotype showed severe GSD symptom compared to indeterminate genotype. Fujii et al. (2015) indicated that earlier flowering genotype showed more severe GSD symptom although this effect was dependent on the allele at the *Dt1* locus, which affects determinate or indeterminate phenotypes. Yamada et al. (2014) detected QTLs which have an effect on GSD occurrences although the function of these QTLs was unknown. Isobe et al. (2015) indicated that the amount of cytokinin in xylem exudate was correlated with GSD severity in a comparison of several cultivars which showed different GSD severity.

Breeding GSD-insensitive cultivars is expected to be an effective solution to GSD. During breeding, comparisons of the GSD severity between many cultivars or lines are needed. However, it is difficult to accurately detect the differences in GSD severity between cultivars or lines because the occurrences of GSD largely vary by field location and cultivation year (Fujii et al. 2015; Hill et al., 2006). In particular, it is generally difficult for breeders to find differences in potential GSD sensitivity between cultivars or

lines when GSD occurrences are somehow suppressed.

Thus, it is desirable to establish an effective experimental treatment that promotes the GSD occurrence in order to stably detect the differences between cultivars or lines.

1.4 Sink removal as an experimental treatment to promote GSD occurrences

Depodding at the pod set and filling stages has been used as an experimental treatment to promote delayed leaf senescence of leaves and stems including GSD (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982). However, depodding is a time- and labor-consuming method and is difficult to do, especially in a field experiment.

In addition to this, depodding has a problem as an experimental technique, because cutting organs induces injury stresses. Turner et al. (2012) indicated that sink limitation by cutting organs in soybean lead to the expression of stress-response genes and the metabolic shifts involved in abiotic or biotic stresses in distant leaves. There is a possibility that GSD promoted by depodding could be affected not only by sink limitation but also by injury stress responses.

It is a problem for sink-source balance hypothesis in study of physiological GSD

mechanism that the hypothesis depends on only the results of sink removal. In order to prove the sink-source balance hypothesis, it must be indicated that enhanced source without reduced sink also promotes GSD occurrences.

Thus, a new experimental treatment which can easily and stably promote GSD is needed for both physiological study of GSD mechanism and breeding GSD insensitive cultivars. Especially, in physiological study of GSD, the treatment of enhanced source without reduced sink is effective to prove sink-source balance hypothesis.

1.5 Objective of this study

The objective of this study is to develop a new method to promote GSD occurrences for studies of GSD mechanism. Based on the sink-source balance hypothesis, it was hypothesized that enhanced source capacity by improved light availability in the middle of growth may promoted GSD occurrences. A thinning treatment as a method to improve light availability in the middle of growth was designed. The thinning treatment involved cutting-off all the above-ground parts of the soybean plant in every other row of the plot, and of every other plant in the remaining rows resulting a quadruple space per plant. Thinning could be thought to improve light availability of remaining plants because

mutual shading of plants was reduced by the treatment.

In chapter 2, whether thinning treatment promote GSD occurrences or not was confirmed in order to show this treatment can be used as a new experimental method to study GSD. Since the thinning treatment assumes that the remaining plant is exposed to enriched light, the specific effects of altered light conditions on GSD occurrences were examined by a shade removal treatment. In this experiment, plants were surrounded by a shade sheet to mimic the shading by neighboring plants and shade sheets were removed at the corresponding time of thinning treatment.

In chapter 3, the sink-source balance hypothesis was verified by using the thinning treatment and shade removal treatment established in chapter 2 in order to elucidate the mechanism of GSD. First, the thinning treatment was applied and analyze a growth parameters and transcriptome of plants after the treatment to confirm whether plants with severe GSD occurrence indicate any physiological symptom of excess source ability such that has been demonstrated in the depodded plants. Second, it was aimed to show the promotion of GSD occurrences by improved light availability corresponds to enhanced source ability by applying several shade removal treatments which is different in duration or timing of shade removal and compare GSD severity or growth parameters among different treatments.

In chapter 4, the effectiveness of the thinning treatment as an experimental method for breeding GSD insensitive cultivars was verified. The thinning treatment was applied on four cultivars, which had been evaluated their GSD severity in the previous tests to confirm (1) whether thinning promotes the GSD occurrences in different cultivars and (2) whether differences in GSD severity between cultivars are clarified by thinning.

In chapter 5, all the results were integrated and the usefulness of the thinning treatment as a new method to study GSD for both physiological mechanism and breeding insensitive cultivars was discussed.

Chapter 2

Effect of thinning and shade removal on green stem disorder in soybean

2.1 Introduction

In order to develop a solution to prevent the occurrence of GSD in soybean, the underlying mechanism of GSD must be elucidated. Toward this, it will be effective to find factors of experimental treatments that promote GSD, and then to analyze the effects of these factors or treatments on soybean physiology and ecology.

Depodding at the pod set and filling stage has been used as an experimental treatment to promote GSD (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1987; Wittenbach, 1982). However, depodding is time-/ labor-consuming method and depodding may have unintended effects on soybean physiology apart from sink limitation because it was reported that cutting sink organs caused stress responses in distant leaves (Turner et al. 2012). Therefore, another experimental treatment to promote GSD occurrences are needed.

In the present study, the author hypothesized that increased light availability to enhance the source-sink ratio would be also promote GSD based on sink-source balance hypothesis. To examine this hypothesis, thinning and shade removal at R1 and R5 stages to alter the light availability was incorporated. Studies on altered light condition have been previously conducted with soybean (Hayati et al., 1995; Mathew et al., 2000; Schou et al., 1978). Hayati et al. (1995) reported the effect of shading from R1 to R5 on the leaf chlorophyll content until R7 stage as the indicator of leaf senescence and discussed that increased photosynthesis did not accelerate leaf senescence. However, they did not mention about GSD occurrences, which must be evaluated by conditions of both leaves and stems at R8 stage. Thus, to our knowledge, this study is the first to examine the relationship between increased light availability and occurrence of GSD in soybean. We also discuss the experimental advantages of thinning in field research compared to other treatments used for studying GSD.

2.2 Materials and methods

2.2.1 Plant materials and experimental site

Two experiments were conducted in the experimental fields of NARO, Western Region

Agricultural Research Center, Hiroshima, Japan (lat. 34°30'N, long. 133°23' E, and 2 m elevation; Typic Fluvaquents soil type) in 2014, 2015, and 2016. The leading soybean cultivar at this site 'Sachiyutaka' was used in all the experiments. Exp. 2.1 during all years were conducted in a field where the ground water level was maintained at 30 cm below the ground surface by a farm-oriented enhanced aquatic system, FOEAS (Wakasugi & Fujimori 2009). Exp. 2.2 was conducted without irrigation. Inorganic fertilizers were applied at 3 g m⁻² of N, 10 g m⁻² of P₂O₅, and 10 g m⁻² of K₂O a day before sowing in all the experiments. Insecticides and fungicides were applied properly to avoid biotic stresses.

2.2.2 Treatments

Exp. 2.1: thinning at R1 or R5 stages

Exp. 2.1 was conducted in 2014, 2015, and 2016. The sowing dates were 25 June 2014, 24 June 2015, and 27 June 2016 (Table 2.1). There were eight plots each (four treatments and two replications) in 2014 and 2015, and four plots (two treatments and two replications) in 2016. The size of each plot was 3.6 m × 3.0 m in 2014, 3.0 m × 3.3 m in 2015, and 3.0 m × 2.1 m in 2016. Planting density was either sparse (0.6 m row and 0.3 m plant spacing; 5.56 plants m⁻²) or dense (0.3 m row and 0.15 m plant spacing; 22.2

plants m⁻²). In addition to the plots in which plant population densities were maintained as sparse or dense from sowing until R8 stage, there were also plots in which plant population density was changed from dense to sparse by performing thinning activities. Thinning involved cutting-off all the above-ground parts of the plant in every other row of the plot, and of every other plant in the remaining rows. In 2014, thinning was conducted on either 11 August or on 11 September, corresponding approximately to R1 and R5 stage (Table 2.1). In 2015, thinning was conducted on 7 August (R1) or 4 September (R5) (Table 2.1). In 2016, there were two plots, in which either thinning was conducted at R5 (5 September), or the plant population density was maintained as dense (Table 2.1). All data were recorded by sampling individual plants randomly selected from each plot and excluding plants on the border of the plot. The number of plants selected was 12 or 11 in 2014, and six in 2015 and 2016. The value for each plot was the average score of the recorded plants, and the mean of the replications was the representative score for each treatment group.

Table 2.1. Dates of growth stage and treatments in Exp. 2.1.

Year	Treatment ¹	The date of the growth stage or treatment					
		Sowing	R1	Thinning at R1	R5	Thinning at R5	R8
2014	Sparse	25 Jun.	9 Aug.	-	5 Sep.	-	25 Oct.
	R1	25 Jun.	10 Aug.	11 Aug.	4 Sep.	-	25 Oct.
	R5	25 Jun.	10 Aug.	-	6 Sep.	11 Sep.	25 Oct.
	Dense	25 Jun.	10 Aug.	-	5 Sep.	-	24 Oct.
2015	Sparse	24 Jun.	4 Aug.	-	31 Aug.	-	22 Oct.
	R1	24 Jun.	5 Aug.	7 Aug.	31 Aug.	-	21 Oct.
	R5	24 Jun.	6 Aug.	-	31 Aug.	4 Sep.	22 Oct.
	Dense	24 Jun.	5 Aug.	-	1 Sep.	-	24 Oct.
2016	R5	27 Jun.	4 Aug.	-	28 Aug.	5 Sep.	2 Nov.
	Dense	27 Jun.	4 Aug.	-	29 Aug.	-	1 Nov.

Note: ¹ Sparse, plant population density was kept at 5.56 plants m⁻². R1, thinning was conducted at R1. R5, thinning was conducted at R5. Dense, plant population density was kept at 22.2 plants m⁻².

Exp. 2.2: shade removal at R1 or R5 stages

Exp. 2.2 was conducted in 2015. The results of Exp. 2.1 indicated that thinning at R5 stage promoted the GSD occurrences. Light intensity is one of the environmental factors modified due to thinning. To examine the specific effects of altered light conditions on GSD, a shading equipment in which plants were surrounded by a shade sheet to mimic the shading by neighboring plants was designed (Fig. 2.1). The equipment was 2.1 m long, 0.4 m wide, and the height was adjustable to match with plants' height. In each plot, seven plants in a single row (plant spacing, 0.3 m) were surrounded by this shade equipment. As the central portion of 0.1 m width on the upper surface of the equipment was opened, a part of uppermost leaves was not shaded to mimic actual field situations where a part of the uppermost leaves of plants in the canopy is not shaded by neighboring plants. The shade sheets were raised as the plants became taller. When one of the uppermost leaves of the plants in the plots became 3 cm taller than the upper side of the shade equipment, the shade sheet was raised immediately above this uppermost leaf. Three types of black colored polypropylene or polyethylene shade sheets were used. Their shading strength were 16%, 82%, and 94% (eliminating 16%, 82%, and 94% of PAR in sunlight on average, respectively). The shading strength of each shade sheet was measured by SunScan Canopy Analysis System – type SS1 (Delta – T Devices Ltd, UK). We covered the PAR

sensor put on the ground with the shade sheet and measured PAR. At the same time, total PAR without any shading was measured by the other PAR sensor. Shading strength was the ratio of PAR covered with shade sheet / total PAR without shading. The scores were the average of 5 measurements for each shade sheet. The sowing date was 24 June 2015 (Table 2.2). There were ten treatment groups with two replications, which differed in the type of shade sheet and the timing of shade removal (Table 2.2). In some plots, the shade sheet was removed on 4 August (R1) or 1 September (R5), corresponding to the date of thinning in Exp. 2.1. In addition, there were the plots shaded until R8 stage or not shaded for the entire growth period (Table 2.2). Data were recorded for every five plants, except two plants at the border of the plot. The value for each plot was the average score of all the plants recorded, and the mean of the replications was the representative score for each treatment group.

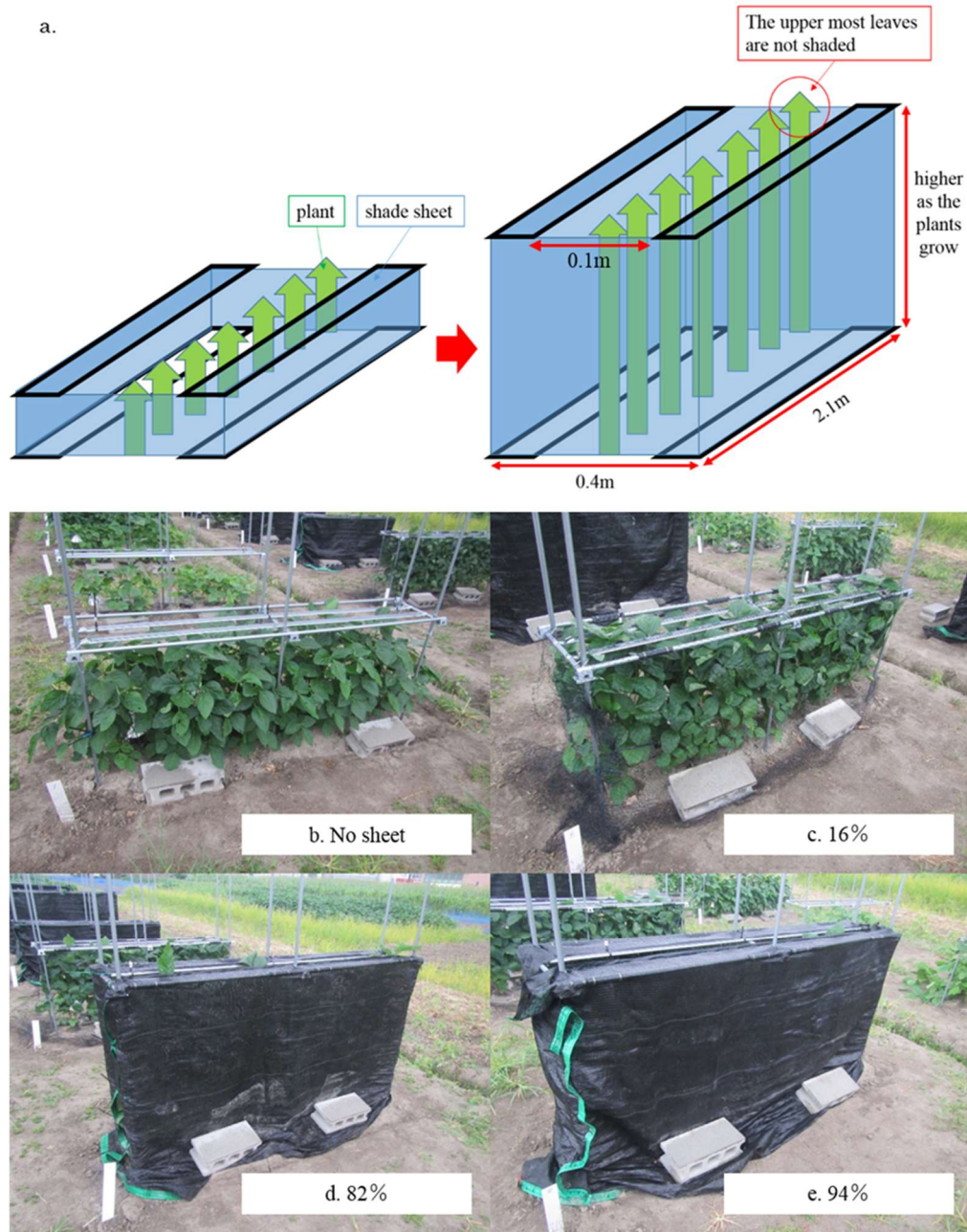


Fig. 2.1. The shade equipment used in Exp. 2.2.

(a) The concept of shading by the shade equipment. The equipment was 2.1 m long and 0.4 m wide. The shade sheet surrounded seven plants planted in a single row, except for their uppermost leaves. The shade sheet can be elongated as the plant grows. (b to e) The appearance of the shade equipment in the experimental field with (b) no sheet, (c) 16% shade sheet (eliminating 16% of PAR from sunlight on average), (d) 82% shade sheet (eliminating 82% of PAR from sunlight on average), and (e) 94% shade sheet (eliminating 94% of PAR from sunlight on average).

Table 2.2. Dates of growth stage and treatment in Exp. 2.2.

Treatment ¹		The date of the growth stage or treatment					
Shade sheet	Stage of shade removal	Sowing	R1	Shade removal at R1	R5	Shade removal at R5	R8
None	-	24 Jun.	3 Aug.	-	28 Aug.	-	25 Oct.
16%	R1	24 Jun.	2 Aug.	4 Aug.	25 Aug.	-	24 Oct.
	R5	24 Jun.	3 Aug.	-	27 Aug.	1 Sep.	25 Oct.
	R8	24 Jun.	3 Aug.	-	27 Aug.	-	25 Oct.
82%	R1	24 Jun.	1 Aug.	4 Aug.	24 Aug.	-	25 Oct.
	R5	24 Jun.	2 Aug.	-	24 Aug.	1 Sep.	17 Oct.
	R8	24 Jun.	2 Aug.	-	24 Aug.	-	18 Oct.
94%	R1	24 Jun.	1 Aug.	4 Aug.	24 Aug.	-	22 Oct.
	R5	24 Jun.	2 Aug.	-	24 Aug.	1 Sep.	18 Oct.
	R8	24 Jun.	2 Aug.	-	24 Aug.	-	20 Oct.

Note: ¹ None means that plants were not surrounded by a shade sheet. 16%, 82%, and 94% mean that plants were surrounded by a shade sheet eliminating 16%, 82%, and 94% of PAR on average, respectively.

2.2.3 Measurements

Following Fehr & Caviness (1977), the dates of growth stages R1, R5, and R8 were recorded for each plant. The severity of GSD was assessed for each plant at R8 stage using the scoring method shown in Table 2.3, adopting the method of Furuya & Umezaki (1993), in which GSD score was assigned based on stem color and the number of green leaves left on the stem. When more than one leaflet was left on the stem, the trifoliate was counted as one green leaf left on the stem. Completely yellowed leaves left on the stem, which was rarely observed, were not counted as green leaves. A high GSD score represents severe GSD symptom in this study, although the severer GSD is, the lower the GSD score is in the study of Furuya & Umezaki (1993). After R8 stage, seed weight, pod number, and the number of total nodes in all branches and main stem were measured. The dry matter N concentration of the main stem was measured using Vario MAX CN (Elementar Analysensysteme GmbH, Germany), except in Exp. 2.1 in 2014. The sixth to eighth nodes or the seventh to ninth nodes counted from the cotyledonary node of the main stem were sampled because the three nodes were approximately at the center of the main stem.

Table 2.3. The scoring method used to evaluate the severity of GSD, following Furuya and Umezaki (1993).

GSD score	The appearance of the plant at R8
5	The stem is green and green leaves remain at more than one-third of all the nodes of the plant.
4	The stem is green and green leaves remain at fewer than one-third of all the nodes of the plant.
3	The stem is green or yellow-green and no green leaves remain.
2	The stem is yellow and retains some moisture, and no green leaves remain.
1	The stem is brown and dry, and no green leaves remain.

Note: GSD score was judged based on the criteria in this table at R8 for each plant. A high score means the plant shows severe GSD symptoms.

2.2.4 Statistical Analysis

All experiments were conducted in a completely randomized design. Analysis of variance (ANOVA) and Tukey's test or t-test were used to test the differences in values and compare the means among the treatment groups ($p < 0.05$ or $p < 0.01$). GSD scores were analyzed after Box-Cox transformation. GSD ratio (ratio of the number of plants with a certain GSD score to that of all plants examined in each plot) were analyzed after angular transformation. Correlation coefficient of GSD score and N concentration of the main stem for each plant sampled was calculated. All the analyses were performed using the statistical software, BellCurve for Excel (Social Survey Research Information Co., Ltd.).

2.3 Results

2.3.1 Exp. 2.1: Thinning at R1 or at R5 stages

Plant appearance at maturity

The appearance of representative plants from Exp. 2.1 in 2014 at maturity is shown in Fig. 2.2. The plants from the continuously dense population (22.2 plants m^{-2}) or the continuously sparse population (5.56 plants m^{-2}) showed no GSD symptoms: all leaves

had fallen and the stems turned brown and dried, even though the number of pods was less, and the stems were thinner and longer in the dense population than in the sparse population. The plants thinned at R1 stage showed GSD symptoms (GSD score 3): yellow-green stems with no leaves. The plants thinned at R5 stage showed severe GSD symptoms (GSD score 4): green stems and some remaining leaves. The plants thinned at R5 stage showed severe GSD symptoms (GSD score 4): green stems and some remaining leaves.

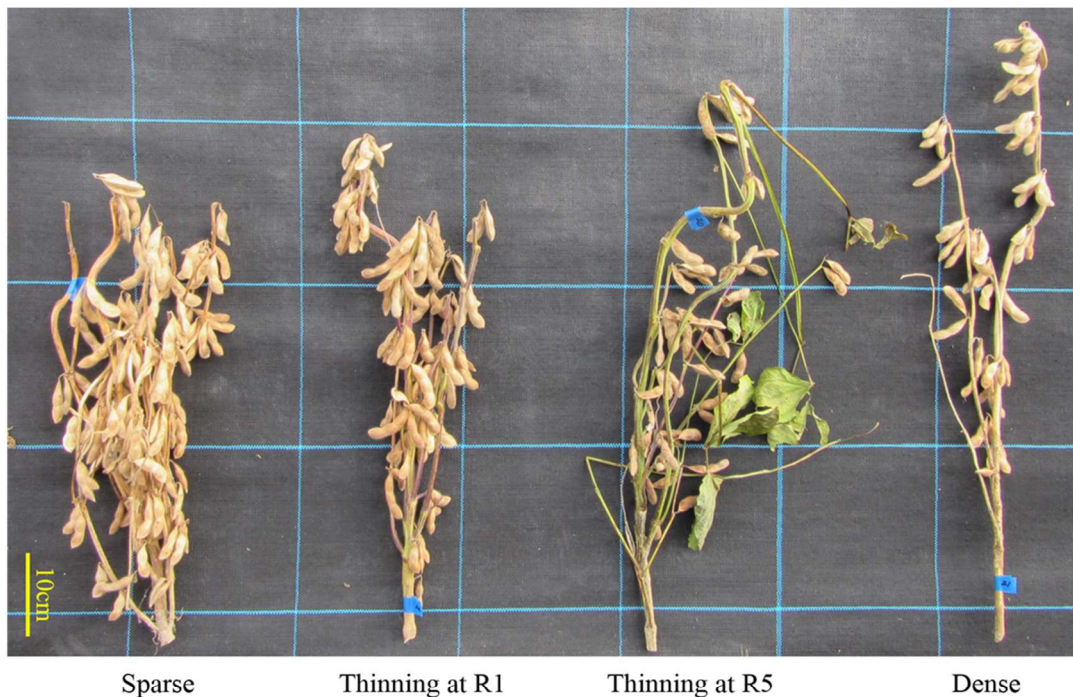


Fig. 2.2. Appearance of representative plants after R8 in the four treatment plots in Exp. 2.1 in 2014.

Sparse means plant population density was kept at sparse ($5.56 \text{ plants m}^{-2}$) from sowing to harvest. Thinning at R1 means plant population density was changed from dense ($22.2 \text{ plants m}^{-2}$) to sparse by thinning on 11 August (R1). Thinning at R5 means plant population density was changed from dense to sparse by thinning on 8 September (R5). Dense means plant population density was kept dense from sowing to harvest.

Severity of green stem disorder

GSD scores of the treatments thinned at growth stage R5 were significantly higher (3.6 to 4.0) than those of the other treatments in the three years (Table 2.4). Mean GSD score in 2014 and 2015 of the treatment thinned at growth stage R1 was significantly higher than those of the treatment in which plant population was kept dense or sparse and was significantly lower than that of the treatment thinned at R5.

GSD ratio (≥ 3) of the treatments thinned at R5 were significantly higher (100.0%) than those of the other treatments in the three years (Table 2.4). GSD ratio (≥ 4) of the treatments thinned at R5 was significantly higher than those of the other treatments in 2014 and in the mean of 2014 and 2015. There were no significant differences in GSD ratio (≥ 5) among any treatments in every year (0 - 4%). The number of leaves remaining at the R8 stage per node, which is one of the measures used to score GSD, showed similar tendency to GSD scores. In Exp. 2.1 in 2015 (Table 2.4), groups thinned at R5 stage (0.119) had significantly higher values than the other groups with a low GSD score (0.000 to 0.004).

Table 2.4. The effects of thinning on GSD and plant characteristics in Exp.2.1

Year	Treatment ¹	GSD score ²	GSD ratio (%) ³			N (mg g ⁻¹) ⁴	Leaf number left at R8 per node ⁵	Seed weight (g plant ⁻¹)	Pod number per node	Total node number per plant
			≥3	≥4	=5					
2014	Sparse	2.4b	39b	5b	0	nd	nd	73a	1.8ab	62a
	R1	2.8b	71b	13b	0	nd	nd	69a	1.9a	55ab
	R5	4.0a	100a	96a	0	nd	nd	33b	1.4bc	35ab
	Dense	2.4b	50b	13b	4	nd	nd	25b	1.2c	34b
	ANOVA	**	**	**	ns	nd	nd	**	*	*
2015	Sparse	2.3b	50b	0b	0	7.2b	0.000b	50a	1.4	71a
	R1	2.8b	67b	8ab	0	7.3b	0.004b	39a	1.6	44b
	R5	3.7a	100a	67a	0	18.5a	0.119a	19b	1.1	27c
	Dense	2.3b	42b	8ab	0	6.0b	0.004b	13b	0.9	29c
	ANOVA	**	**	*	ns	**	**	*	ns	**
mean of 2014 and 2015	Sparse	2.4c	45b	4b	0	nd	nd	62a	1.6ab	67a
	R1	2.8b	69b	16b	0	nd	nd	54a	1.7a	50b
	R5	3.8a	100a	68a	0	nd	nd	26b	1.2bc	31c
	Dense	2.4c	46b	16b	2	nd	nd	19b	1.0c	31c
ANOVA	Treatment	**	**	**	ns	nd	nd	**	**	**
	Year	*	ns	*	ns	nd	nd	**	**	ns
	Interaction	ns	ns	ns	ns	nd	nd	ns	ns	ns
	R5	3.6	100	58	0	11.4	0.06	34	1.8	31
2016	Dense	2.3	25	8	0	3.6	0.002	24	1.4	36
	t-test	**	**	ns	ns	ns	ns	ns	ns	ns

Note: Within columns in each year, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**, Significant at the 0.01 probability level. *, Significant at the 0.05 probability level, ns, nonsignificant at the 0.05 probability level. nd, no data.

¹ Sparse, plant population density was kept at 5.56 plants m⁻². R1, thinning was conducted at R1. R5, thinning was conducted at R5. Dense, plant population density was kept at 22.2 plants m⁻².² ANOVA, Tukey's test, and t-test were conducted after Box-Cox transformation.³ GSD ratio indicates the ratio of the number of plants with GSD score ≥3, ≥4, or =5 to the number of all plants sampled in the plot. ANOVA, Tukey's test, and t-test were conducted after angular transformation.⁴ N (mg g⁻¹) is dry matter N concentration in main stem.⁵ Leaf number left at R8 per node is the number of green leaves left at R8 / total node, which was calculated for each plant.

N concentration in the main stem

In Exp. 2.1 in 2015 (Table 2.4), N concentration of the main stem at maturity in the group thinned at R5 stage was significantly higher than that in the other groups. In Exp. 2.1 in 2016 (Table 2.4), the group thinned at R5 stage also tended to have higher N concentration than that in the dense group ($p = 0.067$). In 2015 and in 2016, N concentration of the main stem showed a significantly positive correlation to GSD scores for each recorded plant (Table 2.5).

Table 2.5. Correlations between GSD score and dry matter N concentration in the main stem for each recorded plant.

Experiment (Year)	Exp.1 (2015) n=24	Exp.1 (2016) n=72	Exp.2 (2015) n=100
r	0.68	0.73	0.68
Significance test	**	**	**

Note: **, Significant at the 0.01 probability level.

Seed weight, pod number per node, and total node number

In Exp. 2.1 (Table 2.4), seed weight tended to increase as the sparse planting period became longer in the three years. Pod number per node and total node number tended to be higher in the group thinned at R1 than the group thinned at R5 in 2014 and in 2015.

2.3.2. Exp. 2.2: Shade removal at R1 or at R5 stages

Severity of green stem disorder

In Exp. 2.2 (Table 2.6), the GSD scores of the treatments of 82% shade removal at R5 and of 94% shade removal at R5 and R8 (4.1 to 4.5) were significantly higher than that of no shading in treatment (2.6). There were no significant differences in GSD scores among the other treatments (2.7 to 3.3) and the no shading treatment. GSD ratio (≥ 4) showed the same tendency as those of GSD score. In GSD ratio (≥ 3), the treatments of 16% shade removal at R5 and of 82% shade removal at R1 also showed significantly higher GSD ratio (100%) than the no shading treatment. There were no significant differences among treatments in GSD ratio (= 5).

In Exp. 2.2 (Table 2.6), the number of leaves remaining at R8 stage per node of the high GSD score groups (82% shade removal at R5 and 94% shade removal at R5 and 94% continuous shading until R8) tended to be higher than those of all other low GSD score

groups.

Table 2.6. The effects of shade removal on GSD and plant characteristics in Exp.2.2.

Treatment		GSD ratio (%) ³			N (mg g ⁻¹) ⁴	Leaf number left at R8 per node ⁵	Seed weight (g plant ⁻¹)	Pod number per node	Total node number per plant
Shade sheet ¹	Stage of shade removal	GSD score ²	≥3	≥4	≥5				
None	-	2.6d	50b	10b	0	4.9cd	150a	2.4abc	98a
16%	R1	2.7cd	70b	10b	0	4.6cd	134ab	2.4bcd	91a
	R5	3.2bcd	100a	20b	0	9.2cd	121ab	2.1cd	82a
	R8	2.7cd	60b	10b	0	6.3cd	106bc	2.1cd	82a
82%	R1	3.3abcd	100a	30b	0	8.8cd	108bc	3.1a	56b
	R5	4.2ab	100a	100a	20	22.4b	47de	2.0cd	43bc
	R8	2.8cd	70b	10b	0	4.1d	38e	1.8de	45bc
94%	R1	2.7cd	70b	0b	0	7.7cd	80cd	2.9ab	42bc
	R5	4.5a	100a	100a	50	29.3a	13e	1.3e	24c
	R8	4.1abc	100a	100a	10	10.3c	14e	1.2e	27c
ANOVA		**	**	**	ns	**	**	**	**

Note: Within columns in each year, means followed by the same letter are not significantly different according to Tukey's test (0.05).

** , Significant at the 0.01 probability level. ns, nonsignificant at the 0.05 probability level.

¹ None means that plants were not surrounded by a shade sheet. 16%, 82%, and 94% mean that plants were surrounded by a shade sheet eliminating 16%, 82%, and 94% of PAR from sunlight on average.

² ANOVA, Tukey's test, and t-test were conducted after Box-Cox transformation.

³ GSD ratio indicates the ratio of the number of plants with GSD score ≥3, ≥4, or =5 to the number of all plants sampled in the plot.

ANOVA, Tukey's test, and t-test were conducted after angular transformation.

⁴ N (mg g⁻¹) is dry matter N concentration in main stem.

⁵ Leaf number left at R8 per node is the number of green leaves left at R8 / total node, which was calculated for each plant.

N concentration in the main stem

In Exp. 2.2 (Table 2.6), N concentrations in the main stem at maturity in the two high GSD score groups shaded until R5 stage with the 82% and 94% shade sheet was 22.4 mg/g and 29.3 mg/g, respectively. These were significantly higher than the values obtained for the other groups (4.1–10.3 mg/g). Although the group shaded until R8 stage with the 94% shade sheet was one of the groups with a high GSD score, this group had lower N concentration (10.3 mg/g) than the other two high GSD score groups.

N concentration of the main stem showed a significantly positive correlation to GSD scores for each recorded plant (Table 2.5).

Seed weight, pod number per node, and total node number

In Exp. 2.2 (Table 2.6), seed weight with no shade sheet during the whole growth period was the highest (150 g /plant⁻¹). The seed weight tended to decrease as the shading period and intensity increased. In any shade sheets, pod number per node and total node number tended to be higher in the group the shade of which was removed at R1 than that at R5 stage.

2.4 Discussion

2.4.1 GSD score and delayed leaf and stem senescence characterize GSD

Harbach et al. (2016) reported that in soybean, delayed senescence symptoms, including GSD, are manifested through various combinations of delayed maturation of the stem, leaves, and pods, but these symptoms have not been adequately distinguished from each other. In the current study, GSD was primarily evaluated using the GSD score, which was based on the color of the stem and the ratio of existing leaves at R8 stage (when almost all pods have matured), as in previous studies (Furuya & Umezaki. 1993; Mochizuki et al. 2005; Tsujimoto et al. 2006; Isobe et al. 2015; Yamada et al. 2014; Fujii et al. 2015; Takehara et al. 2016). In both the current experiments, the developmental progression of the reproductive growth stage (days from sowing to R1, R5, and R8 stages) were not significantly different (Table 2.1 and Table 2.2), suggesting that GSD in our experiments was characterized not by the accelerated maturation of the pods, instead by delayed maturation of the leaves and stems. We also found that the GSD score was positively and significantly correlated to the N concentration of the main stem in all experiments (Table 2.5). Previous studies have shown that depodded GSD soybean plants tend to have high dry matter N concentration in the stem (Egli & Bruening 2006). Taken together the above-

mentioned results suggest that GSD scores properly represent GSD symptom, and that GSD in this study is characterized mainly by delayed leaf and stem senescence.

2.4.2 Improved light availability at R5 stage promotes GSD

Depodding is the most frequently used treatment in research of leaf and stem senescence, including GSD research (Leopold et al. 1959; Mondal et al. 1978; Wittenbach 1982; Crafts-Brandner et al. 1984; Crafts-Brandner & Egli 1987; Egli & Bruening 2006; Htwe et al. 2011), which decreases sink size and then increases the source-sink ratio. In this study, a novel approach was employed to increase the source-sink ratio.

During the three years of Exp. 2.1, thinning at R5 stage following dense cultivation showed a significantly higher GSD score and GSD ratio (≥ 3) than that in a continuously dense cultivation (Table 2.4). In Exp. 2.2, removal of the 82% shade sheet at R5 stage that mimicked improved above-ground light conditions resulting from thinning produced a significantly higher GSD score or higher N concentration in the main stem compared to that in continuous shading until R8 stage (Table 2.6). These results suggest that increased light availability at R5 stage promotes GSD, probably by enhancing the source relative to the sink. This finding just reconfirms the results of previous studies in which the sink was reduced in order to promote GSD (Leopold et al. 1959; Mondal et al. 1978; Wittenbach

1982; Crafts-Brandner et al. 1984; Crafts-Brandner & Egli 1987; Egli & Bruening 2006; Htwe et al. 2011). However, to our knowledge, it has never been reported that the enhanced source also promoted GSD.

2.4.3 Difference in treatments at R1 and R5 stages

In the context of relative increases in source-to-sink, it is also notable that the effects of thinning and shade removal on GSD were significantly stronger at R5 than at R1 stage as was shown in the GSD score in 2014 and 2015 (Table 2.4) and of the GSD ratio (≥ 4) (Table 2.5). Schou et al. (1978) reported that light enrichment before R5 stage resulted in higher yield compared to that after R5 stage. It was reported that source restriction by defoliation or shading determine pod and seed number between R1 and 10 to 12 days after R5 (Board & Tan 1995) or between R1 and 14 to 21 days after R5 (Egli 2010). Thus, one important difference between the plants at R1 and R5 stages is their ability to increase the number of pod per plant in response to improved light environment.

Stephenson & Wilson (1977) demonstrated that carbon assimilated in a leaf basically accumulated in pods in the axil of the leaf. Nobuyasu et al. (2003) showed that the involved leaf and pods collectively formed a sink-source unit with the subtending internode. Therefore, it would be important to consider a sink-source balance on the basis

of the sink-source unit. The number of pod per node at R8 stage can serve as a good estimate for an averaged sink size per sink-source unit after R5 stage, which was higher in the groups thinned or shade-removed at R1 than at R5 stage (Table 2.4 and Table 2.6). Meanwhile, an averaged source ability per sink-source unit after R5 stage would be no higher in the groups treated at R1 than at R5 stage because of mutual shading provided by the leaves themselves. The degree of mutual shading could be rationally estimated based on the total node number per plant, which tended to be higher in the groups treated at R1 than at R5 stage (Table 2.4 and Table 2.6). Therefore, the source-sink ratio based on the sink-source unit after R5 stage could be lower in the group treated at R1 stage than that at R5 stage, leading to differences in GSD between treatments at R1 and R5 stages.

2.4.4 Continuous strong shading also promotes GSD-like symptom

The groups with no change in the light environment tended to have low GSD scores, except under the 94% shade sheet in Exp. 2.2 (Table 2.6). The continuously shaded group with high GSD score also showed a different trend in terms of N concentration in the main stem, with a significantly lower N concentration than that in the other plants with a high GSD score in Exp. 2.2. The reason for this result remains to be elucidated. Strong shading above certain levels (such as the 94% shade sheet, eliminating 94% of PAR) may

also enhance GSD-like symptom without altering the N status, as seen in Exp. 2.2. This result is in line with those of previous studies in which dark incubation of whole Arabidopsis plants delayed senescence, while dark incubation of individual leaves promoted senescence (Weaver & Amasino 2001; Rolland et al. 2006). Although the underlying mechanism of delayed senescence in strongly shaded soybean plants and dark-incubated Arabidopsis plants needs to be elucidated, the mechanism described seems to be different from that in naturally occurring GSD.

2.4.5 Thinning and shade removal as experimental methods to reproduce GSD symptoms

The treatment used in this study, particularly thinning, is a simpler, less time/labor consuming method than depodding, and is especially valuable for field experiments and elucidating the precise mechanism of GSD. Depodding causes injury stresses due to the cutting of pods, whereas thinning or shade removal does not. Turner et al. (2012) reported that sink limitation by cutting organs in soybean induces the expression of stress-response genes and the metabolic shifts involved in abiotic or biotic stresses in distant leaves. They suggested considering the unintended consequences due to cutting the organs.

Thinning and shade removal may also be useful for breeding GSD insensitive cultivars.

In breeding and QTL analysis, many comparisons of the occurrence of GSD in each line or cultivar are needed. However, it is difficult to accurately identify GSD insensitive lines and cultivars, because the occurrence of GSD largely varies by location and year (Hill et al. 2006; Fujii et al. 2015). Given that thinning and shade removal can stably promote the occurrence of GSD, these treatment techniques may be useful as a means to produce GSD symptoms in experimental lines and cultivars, particularly in localities or during years with extremely low occurrence of GSD.

Chapter 3

Effect of improved light environment on green stem disorder in soybean corresponded to enhanced source ability

3.1 Introduction

In chapter 2, it was revealed that the thinning and the shade removal treatment at R5 growth stage promoted GSD occurrences. Thus, the thinning treatment and shade removal treatment could be new and useful experimental methods for physiological study on GSD mechanism. In this chapter, I attempted to elucidate the physiological mechanism how these treatments promote GSD occurrences.

As was discussed in chapter 2, improvement in light availability by thinning and shade removal promoted GSD occurrences. These results could indicate that enhanced source ability without reduced sink size promoted GSD occurrences, which would strongly support the sink-source balance hypothesis. However, whether improved light availability enhanced source ability or not has not been shown in chapter 2. Thus, the objective of this chapter is to answer that question in order to verify the sink-source balance hypothesis.

Firstly, whether plants treated with improved light availability show growth and physiological feature which indicate excess source was confirmed. In the point of growth parameters, it was reported that the deflowering treatments after R1 growth stage increased dry matter weight of leaf blades and stems after 10 days from R1, although deflowering had only a minimal effect on the leaf area, the number of branches, and the number of branch nodes (Heitholt & Egli, 1985). In that study, vegetative plants served as sinks for excess source by deflowering. In the point of physiological parameters, Brown & Hudson (2017) conducted transcriptome analysis of both depodded plants and male-sterile plants to identify transcriptional changes of sink-limited plants. As a result, Gene Ontology-terms (GO-terms) associated with growth and development and storage proteins were over-represented in genes that were differentially expressed between sink-limited and control plants. In this chapter, growth parameters and transcriptome of light improved plants were analyzed to confirm whether these plants show same tendency to indicate excess source as depodded or male-sterile plants as shown in the previous studies. As a method to improve light availability, I chose the thinning treatment because thinning can be easily conducted and treated enough plants for destructive analysis. The timing of focus for sampling was set at 28 days after R5 (R5+28d), because plants at R5+28d do not show yellowing of monocarpic senescence yet and can be thought appropriate for

analyzing growth and transcriptome.

Secondly, whether level of enhanced source corresponds to GSD severity or not was confirmed. To confirm this, I applied the shade removal treatment because shade equipment can change shading condition reversibly. The level of enhanced source by changing duration and timing of shade removal was modified and the effect of duration and timing of shade removal on GSD severity was analyzed. It was hypothesized that GSD severity become higher as duration of improved light availability after R5 become longer, or GSD severity become lower as timing of improved light availability become later. That is because longer duration of improved light availability caused increase in photosynthesis and later timing of improved availability has lower effect of increase in photosynthesis.

In this chapter, I attempted to elucidate (1) whether thinned plants show same growth and gene expression pattern which indicate excess source ability as depodded plants, and (2) whether duration and timing of enhanced source by shade removal correspond to GSD severity, to measure validity of sink-source balance hypothesis using two treatments of improved light availability.

3.2 Materials and methods

3.2.1 Plant materials and experimental site

Two experiments (Exp. 3.1 and Exp. 3.2) were conducted in the experimental field of NARO, Western Region Agricultural Research Center, Hiroshima, Japan (lat. 34°30'N, long. 133°23' E, and 2 m elevation; Typic Fluvaquents soil type). The ground water level in all experimental fields was maintained at 30 cm below the ground surface by the farm-oriented enhanced aquatic system FOEAS (Wakasugi & Fujimori, 2009). Inorganic fertilizers were applied at 3 g m⁻² of N, 10 g m⁻² of P₂O₅, and 10 g m⁻² of K₂O a day before sowing in all the experiments. Insecticides and fungicides were applied properly to avoid biotic stresses. The leading soybean cultivar at this site 'Sachiyutaka' was used in all the experiments.

3.2.2 Treatments

Exp. 3.1 Effects of thinning on transcriptome and growth parameters of vegetative organs

Exp. 3.1 was conducted in 2016 and in 2018. The sowing dates were 27 June 2016, and 25 June 2018. There were two treatments and two replications in 2016 and two treatments

and four replications in 2018. The size of each plot was 3.0 m × 1.2 m in 2016 and 3.0 m × 2.1 m in 2018. There were two kinds of treatment plots, thinning treatment plot and dense treatment plot. Planting density was dense (0.3 m row and 0.15 m plant spacing; 22.2 plants m⁻²) until R5 in every plot. In the thinning treatment plots, planting density was altered from dense to sparse (0.6 m row and 0.3 m plant spacing; 5.56 plants m⁻²) by thinning at R5 growth stage. Thinning involved cutting-off all the above-ground parts of the plant in every other row of the plot, and of every other plant in the remaining rows. In the dense treatment plots, planting density was maintained as dense plant population until R8 growth stage.

Exp. 3.2: Effects of duration and timing of thinning on GSD

Exp. 3.2 was conducted in 2016, in 2017, and in 2018. The sowing dates were 22 June 2016, 28 June 2017, and 29 June 2018. In each plot, seven plants in a single row (plant spacing, 0.3 m) were surrounded by a shade equipment, which was used in chapter 2. The equipment was 2.1 m long, 0.4 m wide, and the height was adjustable to match with plants' height. As the central portion of 0.1 m width on the upper surface of the equipment was opened, a part of uppermost leaves was not shaded to mimic actual field situations where a part of the uppermost leaves of plants in the canopy is not shaded by neighboring plants.

The shade sheets were raised as the plants became taller. When one of the uppermost leaves of the plants in the plots became 3 cm taller than the upper side of the shade equipment, the shade sheet was raised immediately above this uppermost leaf. The shade sheets used in the equipment was black colored polypropylene sheets, whose shade strength was 82% (eliminating 82% of PAR in sunlight on average). That is same as one of the three kind of shade sheets in chapter 2. There were six treatment groups with two (in 2016 and in 2017) or four (in 2018) replications, which differed in the duration or the timing of shade removal (treatment 1 to treatment 6 in Fig. 3.1). In treatment 1, shading was maintained from sowing to harvest. In treatment 2, 3, and 4, shade sheet was removed at R5 growth stage. Shading was resumed at 14 days after R5 in treatment 2, and 28 days after R5 in treatment 3. In treatment 5, shade sheet was removed at 28 days after R5. In treatment 6, shade sheet was removed at 42 days after R5. The treatment 7 in Fig. 3.1, in which there was no shade sheet from sowing to harvest, was conducted except in 2016.

Treatment	Shade removal		Sowing	R5	R5+14d	R5+28d	R5+42d	R8
	Duration (weeks)	Timing						
1	0	-						
2	2	R5						
3	4	R5						
4	8	R5						
5	4	R5+28d						
6	2	R5+42d						
7	16	-						

: Shading (with shade sheets)
 : Shade removal (without shade sheets)

Fig. 3.1 Duration and timing of shade removal in Exp. 3.2.

3.2.3 Measurements

In all experiments, the dates of growth stages R5 and R8 were recorded for each plant, following the method of Fehr & Caviness (1977). The severity of GSD was assessed for each plant at the R8 stage using a scoring method slightly modified from that of Furuya & Umezaki (1993) (chapter 2). In the scoring method, the GSD score (1-5) was assigned based on the stem color and number of leaves left on the stem at R8. A high GSD score represents severe GSD symptoms. The number of leaves left at R8 was also recorded. After R8 growth stage, seed weight, the number of seeds, the number of pods, and the number of nodes were measured for each plant sampled. 100 seeds weight was calculated by seed weight and seed number for each plant. Dry weight of stem was measured after drying at 80°C for 3 days.

For growth analysis of Exp. 3.1 in 2018, nine plants of each of the dense and the thinning treatment except border plants were randomly selected and sampled with four (in 2018) replicates at R5 (treatment start), 28 days after treatment R5 (R5+28d), and R8 for analysis of growth parameters. Leaf area, the number of leaves, dry weight of leaflets, dry weight of stem, dry weight of leaf petioles, and dry weight of pods and seeds were measured for each plant sampled at R5, R5+28d, and R8. Leaf area was measured using a LI-3100C (LI-COR). Dry weight of each part of plant was measured after drying at 80°C for 3 days.

For transcriptome analysis of Exp. 3.1 in 2016, three main stems of each of the dense and thinning treatment at 14 days after treatment R5 (R5+14d) and 28 days after treatment R5 (R5+28d) were sampled with two field replicates and frozen in liquid nitrogen (LN₂) for storage at -80°C freezer in 2016. Stems were cut with scissors, wrapped with aluminium foil and dipped into LN₂. Stems were completely crushed using the multi-beads shocker (YASUI KIKAI, Japan). Total RNA was prepared using an RNeasy Plant Mini Kit (Qagen, MD, USA). During RNA extraction the DNase treatment was not applied. The concentration of total RNA was measured using a NanoDrop (Thermo Fisher Scientific, MA, USA) and adjusted to 100 ng/μl by dilution. The quality was checked for integrity before performing the RNA sequencing process using the Bioanalyzer (Agilent Technologies, CA, USA). For library preparation, 500 ng of total RNA per sample was used. The library construction was carried out as previously described (Nagano et al., 2015). Sequencing was carried out on each library to generate 100 bp PE reads for transcript sequencing on an Illumina High-Seq 4000 platform by a commercial service provider (Macrogen, Kyoto, Japan). Raw data from RNA-Seq was processed to remove adapter sequences and low quality bases by trimmomatic-0.30 with the option “ILLUMINACLIP:adapter.fa:2:30:10 LEADING:15 TRAILING:15 SLIDINGWINDOW:4:15 MINLEN:32” (Bolger et al. 2014). Then

trimmed paired reads were mapped on the soybean genome reference version 2.0

(Gmax275:

<http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=Phytozome>

#, downloaded on May 15, 2015) using STAR version 020201 with the option “--

alignIntronMax 10000 --outFilterMultimapNmax 20 --outFilterMatchNmin 0 –

outFilterMatchNminOverLread 0.3 --outFilterScoreMinOverLread 0.3

--genomeLoad LoadAndKeep --limitBAMsortRAM 50000000000 --outSAMtype

BAM SortedByCoordinate --outWigStrand Unstranded --outSAMstrandField

intronMotif”. Mapped reads were then passed onto Cufflinks for transcript assembly

(Nagano et al. 2015). For the transcriptome experiments in Exp. 3.1, quantification and

normalization were performed using the SeqMonk software (v1.38.2)

(<http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/>) and Java (1.8.0_151).

Briefly, gene expression was quantitated with RNA-Seq pipeline quantitation on merged

transcripts counting reads over exons correcting for feature length. The data was log

transformed. 54878 probes were generated. For the R5+14d datasets, only the first field

replicate was used for analysis because of low RNA quality of the second replicate. For

the R5+28d datasets, the two field replicates were grouped. The probes were filtered by

Intensity Difference test with $p < 0.05$ and then further screened by a value above -2.0.

The filtered probes were analyzed by gene ontology (GO) term analysis at the web service of SoyBase (<https://www.soybase.org/>) with annotations for the Wm82. a2. v1 (Glyma 2.0).

In Exp. 3.1, six plants were sampled to assess GSD severity by GSD score at the R8 growth stage. In Exp.2, five plants except border of the plot were sampled in 2016, and seven plants of the plot were sampled in 2017 and in 2018 at the R8 growth stage.

3.2.4 Statistical analysis

All experiments were conducted in a completely randomized design. Analysis of variance (ANOVA) and Tukey's test were used to test the differences in values and compare the means among the treatment groups or years ($p < 0.05$ or $p < 0.01$). GSD scores were analyzed after Box-Cox transformation. The values of leaf number left at R8 / total nodes number were analyzed after angular transformation. The analyses were performed using the statistical software, BellCurve for Excell (Social Survey Research Information Co., Ltd.).

3.3 Results

Exp. 1 Effects of thinning on transcriptome and growth parameters of vegetative organs

3.3.1 Severity of GSD in Exp. 1

The GSD score of thinning treatment at R5 (3.4) was significantly higher than dense treatment (1.9) in the average of two-year experiment (Table 3.1). There was no significant difference in GSD score between two years and no significant interaction between treatment and year (Table 3.1). There was no difference between treatments in dates of R8, although there was significant difference between year (Table 3.1). These results showed same tendency as the equivalent experiments conducted in 2014 and in 2015 (chapter 2).

Table 3.1. Effect of thinning on GSD in Exp. 3.1.

Treatment and Year		GSD score	Leaf number left at R8/ Total node number	R8 (DAS)
Treatment ¹	Dense	1.9	0.001	130
	Thinning	3.4	0.023	129
Year	2016	2.6	0.005	125
	2018	2.7	0.015	132
ANOVA ²	Treatment	**	**	ns
	Year	ns	**	**
	Interaction	ns	*	ns

Note: **, Significant at the 0.01 provability level. *, Significant at the 0.05 probability level. ns, nonsignificant at the 0.05 probability level.

¹Dense, plant population density was kept at 5.56 plants m⁻². Thinning was conducted at R5.

²ANOVA was conducted after Box-Cox transformation.

3.3.2 Growth parameters of vegetative organs at R5, R5+28d, and R8

There were no significant differences between treatment in leaflet dry weight, stem dry weight, leaf area, which indicate the amount of vegetative organ, before thinning treatment (R5) (Fig. 3.2a, b, and c). At 28 days after thinning treatment (R5+28d) and at R8, these all parameters were significantly higher in the thinning treatment than in dense treatment (Fig. 3.2a, b, and c). In the dense treatment plants, these all parameters decreased after R5 (Fig. 3.2a, b, and c). On the contrary, in the thinning treatment plants, leaflet dry weight and stem dry weight increased between R5 and R5+28d (Fig. 3.2a and b), although leaf area decreased (Fig. 3.2c). Leaf dry weight per leaf area was significantly higher in the thinning treatment plants than in the dense treatment plants at R5+28d (Fig. 3.2d).

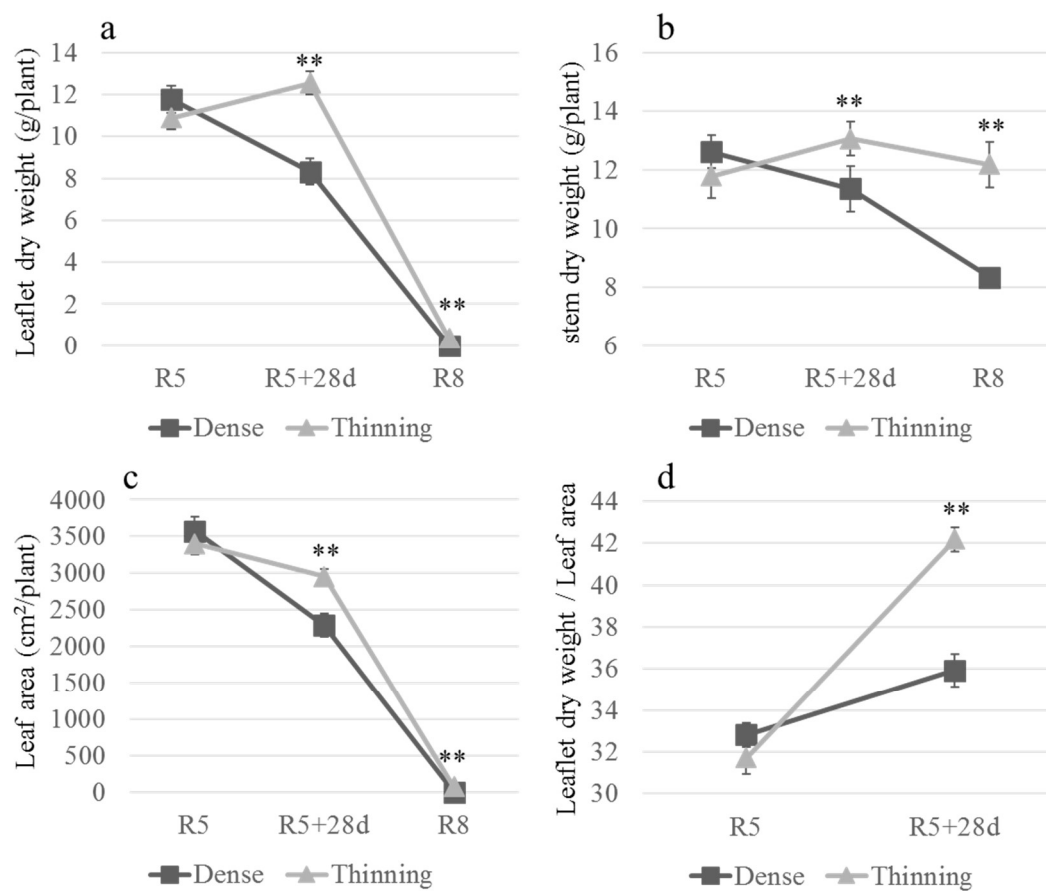


Fig. 3.2 Effect of the thinning treatment at R5 on the parameters which indicate amount of vegetative organ at R5, R5+28d, and R8.

3.3.3 Effect of thinning on transcriptome of main stem at R5+14d and R5+28d

Total 54878 probes were filtered by intensity difference test of SeqMonk at p-value less than 0.05 to highlight the significantly upregulated or downregulated genes by thinning (211 and 528 probes for R5+14d and R5+28d datasets, respectively) (Fig. 3.3). The filtered probes were further screened by a quantified value above -2.0 (126 and 132 probes for R5+14d and R5+28d datasets, respectively) because the scatter plots of expression between the dense and the thinning treatments showed a different distribution far away from the line of $y=x$ under a low expression level (Fig. 3.3).

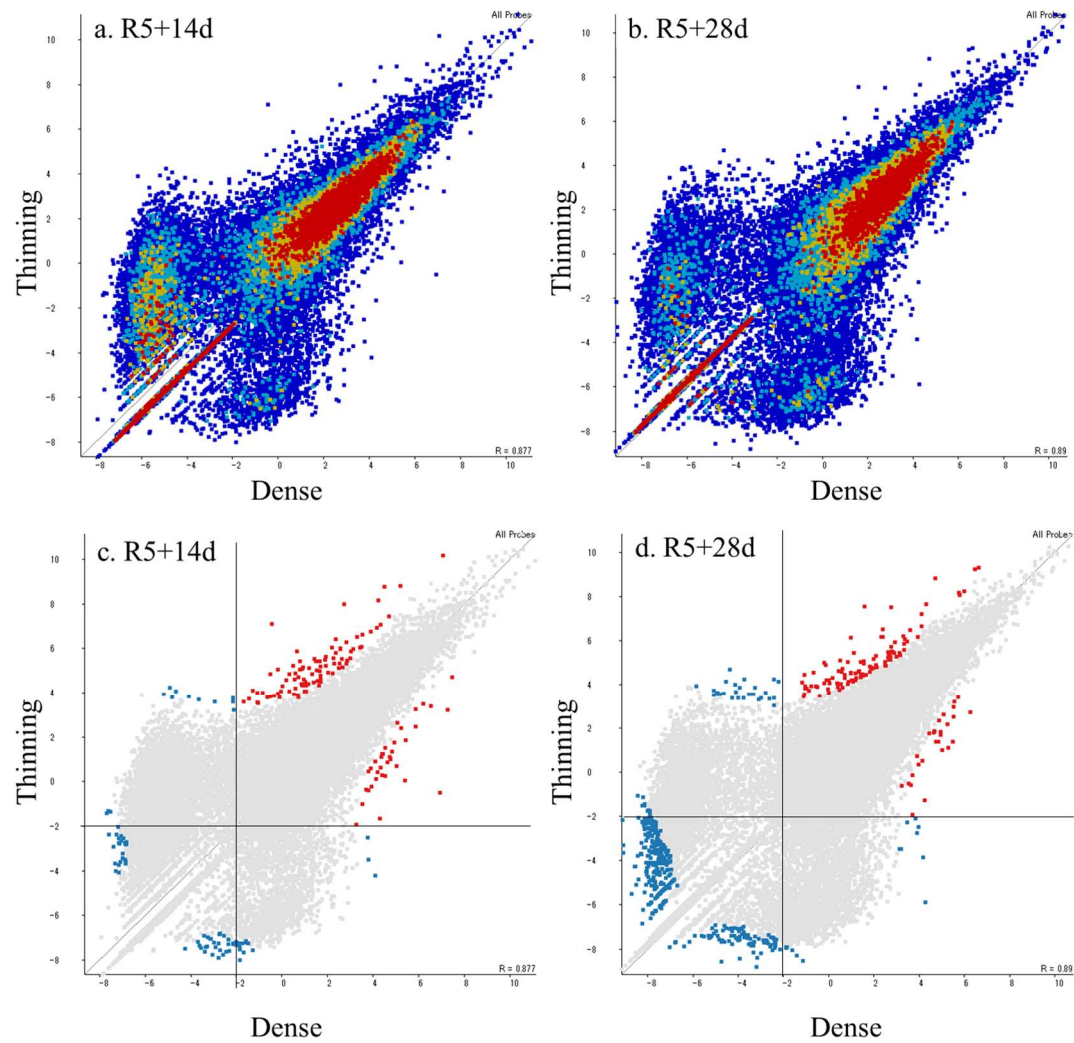


Fig. 3.3 Scatter plots of probes quantified with RNA-seq

Horizontal axis means relative gene expression level of plants of dense treatment ($\log_2$ scale). Vertical axis means relative gene expression level of plants of thinning treatment ($\log_2$ scale). One plot indicates one probe.

(a, b) Scatter plot of all probes at R5+14d (a) and at R5+28d (b).

(c, d) Filtered probes at R5+14d (c) and at R5+28d (d). Dark colored plots above the horizontal line ($y = -2$) and the vertical line ($x = -2$) indicates the probes screened by intensity difference of p-value less than 0.05 and filtered by a quantified value above -2.0.

The GO term analysis of the filtered and screened probes gave a similar ontology for biological process between the R5+14d and R5+28d datasets. 29 and 41 genes had GO-term at R5+14d and R5+28d, respectively (Table 3.2). The results indicated that genes involved in photosynthesis were the most overrepresented genes in upregulated or downregulated genes by thinning treatment at R5 (Table 3.2). However, in the GO term analysis of almost half of all probes (26836 probes) filtered by a quantified value above -2.0 ($\log_2$ scale), photosynthetic genes were not the most overrepresented genes (Table 3.3).

Table 3.2 GO-term analysis of significantly upregulated or downregulated genes by thinning treatment at R5.

Datasets	GO-term	The number of genes	ratio (%)
R5+14d	Photosynthesis	13	44.8
	Carbohydrate Metabolic Process	7	24.1
	Biosynthetic Process	2	6.9
	Growth	2	6.9
	Lipid Metabolic Process	1	3.4
	Transport	1	3.4
	Signal Transduction	1	3.4
	Multicellular Organismal Development	1	3.4
	Cellular Metabolic Process	1	3.4
total		29	100
R5+28d	Photosynthesis	23	56.1
	Carbohydrate Metabolic Process	4	9.8
	Cell Differentiation	3	7.3
	Growth	3	7.3
	Biosynthetic Process	2	4.9
	Multicellular Organismal Development	2	4.9
	Cell Growth	1	2.4
	Cellular Metabolic Process	1	2.4
	Translation	1	2.4
	Nucleobase, Nucleotide, Nucleotide and Nucleic Acid Metabolic Process	1	2.4
total		41	100

Table 3.3 GO-term analysis of genes filtered by a quantified value above -2.0.

Datasets	GO-term	The number of genes	ratio (%)
R5+14d	Signal Transduction	610	13.9
	Translation	597	13.6
	Transport	522	11.9
	Carbohydrate Metabolic Process	392	8.9
	Photosynthesis	316	7.2
	Cell Growth	263	6.0
	Lipid Metabolic Process	193	4.4
	Biosynthetic Process	192	4.4
	Cell Differentiation	189	4.3
	Embryo Development	174	4.0
	Flower Development	139	3.2
	Multicellular Organismal Development	131	3.0
	Growth	107	2.4
	Cell Death	103	2.3
	Cellular Metabolic Process	83	1.9
	Post-embryonic Development	74	1.7
	Protein Modification Process	68	1.6
	Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Metabolic Proc	58	1.3
	DNA Metabolic Process	52	1.2
	Developmental Process	34	0.8
	Cellular Protein Metabolic Process	32	0.7
	Reproductive Structure Development	27	0.6
	Cellular Process	23	0.5
	Macromolecule Modification	5	0.1
	Cellular Developmental Process	2	0.0
	Primary Metabolic Process	1	0.0
total		4387	100
R5+28d	Translation	594	14.0
	Signal Transduction	581	13.7
	Transport	505	11.9
	Carbohydrate Metabolic Process	363	8.6
	Photosynthesis	292	6.9
	Cell Growth	257	6.1
	Cell Differentiation	192	4.5
	Biosynthetic Process	183	4.3
	Lipid Metabolic Process	174	4.1
	Embryo Development	171	4.0
	Flower Development	138	3.3
	Multicellular Organismal Development	129	3.0
	Growth	103	2.4
	Cell Death	102	2.4
	Cellular Metabolic Process	82	1.9
	Post-embryonic Development	78	1.8
	Protein Modification Process	70	1.6
	Nucleobase, Nucleoside, Nucleotide And Nucleic Acid Metabolic Proc	58	1.4
	DNA Metabolic Process	52	1.2
	Developmental Process	35	0.8
	Cellular Protein Metabolic Process	29	0.7
	Reproductive Structure Development	27	0.6
	Cellular Process	23	0.5
	Macromolecule Modification	5	0.1
	Cellular Developmental Process	2	0.0
total		4245	100

The filtered and screened probes were then compared between the R5+14d and R5+28d datasets, identifying the common upregulation of 19 genes and the common downregulation of 8 genes (Table 3.4). The commonly upregulated genes (gene products) include Photosystem II 22 kDa protein, Chlorophyll a/b-binding protein CP24, Chloroplast pigment-binding protein CP26, Early light-induced protein, Stem 28 kDa glycoprotein (Vegetative Storage Protein A), Stem 31 kDa glycoprotein (Vegetative Storage Protein B), and Lipoxygenase, which are photosynthesis-related genes or those which were previously identified to be upregulated in leaves of sink-limited soybeans (Brown & Hudson, 2017).

Table 3.4 Genes commonly upregulated or downregulated by the thinning treatment at R5 in both R5+14d and R5+28d datasets.

Expression	Gmax 2.0 Primary Protein ID	Top Descriptive Uniref100 BLASTP Hit
Upregulated	Glyma.04G249700	Photosystem II 22 kDa protein
	Glyma.06G113200	Photosystem II 22 kDa protein
	Glyma.06G143700	Potassium transporter
	Glyma.06G301500	Beta-amylase
	Glyma.07G014500	Stem 28 kDa glycoprotein VSPA
	Glyma.08G074000	Chlorophyll a/b-binding protein CP24
	Glyma.08G138200	Myo-inositol-1-phosphate synthase
	Glyma.08G180000	Chlorophyll a/b-binding protein CP24
	Glyma.08G200100	Stem 31 kDa glycoprotein VSPB
	Glyma.09G154700	Chloroplast pigment-binding protein CP26
	Glyma.10G243800	Early light-induced protein
	Glyma.12G102900	Beta-amylase
	Glyma.12G217300	ADR6 protein
	Glyma.12G217400	Sali3-2
	Glyma.15G026400	Lipoxygenase
	Glyma.15G026500	Lipoxygenase
	Glyma.15G052400	Chlorophyll a/b-binding protein CP24
	Glyma.18G057900	Dihydroflavonol reductase
	Glyma.20G150600	Early light-induced protein
Downregulated	Glyma.03G226000	Endo-1,4-beta-mannanase
	Glyma.06G136600	Two-component response regulator-like PRR73
	Glyma.07G043000	Dormancy/auxin associated family protein
	Glyma.13G322500	Xyloglucan endotraglucosylase/hydrolase
	Glyma.15G186100	Cytidine/deoxycytidylate deaminase
	Glyma.15G228300	NA
	Glyma.16G043200	NAC transcription factor
	Glyma.19G223000	Endo-1,4-beta-mannanase

note: Upregulated or Downregulated means gene expression level was significantly upregulated or downregulated in the thinning treatment plot compared to the dense treatment plot.

Exp. 2 Effects of duration and timing of shade removal on GSD and growth parameters

3.3.4 Severity of GSD in Exp. 2

The treatment groups of shade removal after R5+28d (treatment 5 and treatment 6 in Fig. 3.1) were not significantly different from treatment 1 (no shade removal) in GSD score (Table 3.5). In the treatment groups of shade removal at R5 (treatment 2, 3, and 4 in Fig. 3.1), GSD score became higher (3.5, 4.3, and 4.6) as the duration of shade removal became longer (14 days after R5, 28 days after R5, and from R5 to R8). However, GSD score of treatment 2 (3.5) was significantly lower than that of treatment 1 (3.9), although duration of shade removal was longer in treatment 2 than in treatment 1 (Table 3.5). Compared to treatment 1 (122 DAS), treatment 3 and 5 took significantly longer (126 DAS) and shorter (116 DAS) time to reach R8 growth stage, respectively (Table 3.5).

In two-year results, GSD score of treatment 1, with shade sheet from sowing to harvest (3.9) was significantly higher than that of treatment 7, without shade sheet from sowing to harvest (2.6). There were no significant differences between treatment 1 and treatment 7 in required days to R8 (Table 3.6).

The number of leaves left at R8 without falling per node showed completely same tendency as GSD score (Table 3.5, Table 3.6).

Table. 3.5 Effect of duration and timing of shade removal on GSD

Treatment and Year		GSD score	Leaf number left at R8/ Total node number	R8 (DAS)
Treatment	1	3.9bc	0.16bc	122bc
	2	3.5d	0.07d	121bc
	3	4.3ab	0.28ab	126a
	4	4.6a	0.34a	123ab
	5	3.8cd	0.12cd	116d
	6	3.9cd	0.16cd	120c
Year	2016	4.2a	0.26a	119b
	2017	3.7b	0.11c	121a
	2018	4.0a	0.19b	122a
ANOVA	Treatment	**	**	**
	Year	**	**	**
	Interaction	ns	ns	*

Note: Within columns, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**, Significant at the 0.01 probability level. *, Significant at the 0.05 probability level. ns, nonsignificant at the 0.05 probability level.

ANOVA was conducted after Box-Cox transformation in GSD score.

Table 3.6 Effect of shading from sowing to harvest on GSD

Treatment and Year		GSD score	Leaf number left at R8/ Total node number	R8 (DAS)
Treatment	1	3.9	0.13	123
	7	2.6	0.00	124
Year	2017	3.2	0.04	123
	2018	3.3	0.08	124
ANOVA	Treatment	**	**	ns
	Year	ns	ns	ns
	Interaction	ns	ns	ns

Note: **, Significant at the 0.01 probability level. *, Significant at the 0.05 probability level. ns, nonsignificant at the 0.05 probability level.

ANOVA was conducted after Box-Cox transformation in GSD score.

3.3.5 Seed weight, seed number, 100 seeds weight, and stem dry weight in Exp. 2

As was the case with GSD score, the treatment groups of shade removal after R5+28d (treatment 5 and treatment 6 in Fig. 3.1) were not significantly different from treatment 1 (no shade removal) in seed weight, seed number, 100 seeds weight, and stem dry weight (Table 3.7). There were no significant differences among all treatments in total node number (data not shown). Compared to treatment 1, seed weight and seed number were significantly higher in treatment 3 (shade removal for 28days after R5) and treatment 4 (shade removal from R5 to R8) (Table 3.7). In treatment 2 (shade removal for 14 days after R5), only seed number was significantly higher than that of treatment 1, although the seed weight showed an intermediate score between treatment 1 and treatment 3 or 4 (Table 3.7). Only treatment 2 showed significantly lower 100 seeds weight than that of treatment 1 (Table 3.7). Stem dry weight became higher as duration of shade removal became longer, that was shown in treatment 1, 2, 3, and 4 in Table 3.7.

Table 3.7 Effect of duration and timing of shade removal on seed weight, seed number, 100 seeds weight, and stem dry weight.

Treatment and Year		Seed weight (g/plant)	Seed number (/plant)	100 seeds weight (g)	Stem dry weight (g)
Treatment	1	27.0cd	77.5c	33.6a	10.4c
	2	31.9bc	96.4ab	30.9b	11.6bc
	3	28.6a	108.4a	33.3a	13.7b
	4	34.5ab	99.7a	31.6ab	17.2a
	5	26.0cd	78.5bc	32.3ab	11.5bc
	6	24.3d	70.4c	32.6ab	10.6c
Year	2016	27.3b	70.0b	30.0c	15.3a
	2017	31.0ab	97.1a	31.8b	10.5c
	2018	31.7a	93.4a	33.8a	12.1b
ANOVA	Treatment	**	**	**	**
	Year	*	**	**	**
	Interaction	ns	ns	*	ns

Note: Within columns, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**, Significant at the 0.01 probability level. *, Significant at the 0.05 probability level. ns, nonsignificant at the 0.05 probability level.

3.4 Discussion

3.4.1 GSD plants thinned at R5 growth stage showed growth features suggesting enhanced source ability

In Exp. 3.1, thinning treatment significantly increased GSD severity (Table 3.1). That indicated the growth features could be compared between GSD plants and non-GSD plants at R5+14d and at R5+28d.

In the present study, the thinning treatment significantly increased leaf area, leaf dry weight, and stem dry weight at R5+28d (Fig. 3.2). Leaf dry weight and stem dry weight were increased from R5 to R5+28d in the thinned plants, although these parameters decreased in the plants of dense treatments. Increased leaf dry weight from R5 to R5+28d in thinned plants was resulted from not only suppression in decrease of leaf area but also increase in specific leaf weight. Heitholt & Egli (1985) reported that sink limited soybean plants showed increase in the mass of the vegetative organs, which was resulted primarily from an increase in specific leaf weight and thicker stems and branches. They discussed that the results suggested vegetative plant parts served as sinks for excess photosynthate when reproductive sinks were limited. In the present study, thinned plants showed same growth features of vegetative parts as sink-limited plants. This suggested that excess

photosynthesis was caused by thinning treatment although reproductive sinks were not limited.

3.4.2 Transcriptome analysis also indicated enhanced source ability

As a result of transcriptome analysis of main stems using RNA-seq in Exp. 3.1, the thinned plants showed overrepresented upregulation of photosynthetic genes (Table 3.2 and Table 3.3) and upregulation of genes of vegetative storage proteins (VSPA, VSPB, and lipoxigenases) (Table 3.4).

These results showed same tendency as the results of previous study, in which transcriptome analysis of depodded soybeans and male-sterile soybeans using RNA-seq was carried out (Brown & Hudson, 2017). That study was conducted to identify transcriptional changes in leaves of sink-limited plants. Male sterile soybeans also showed a delay in leaf senescence like depodded and thinned soybean (Burke et al. 1984). In that study, genes with GO-term of photosynthesis were overrepresented in commonly upregulated genes between depodded and male-sterile plants. Upregulation of vegetative storage protein genes including the same genes in the present study (VSPA, VSPB, and lipoxigenases) could be seen. VSPA, VSPB and lipoxigenases were reported in several studies as the evidence of excess source ability by depodding (Staswic, 1988, Wittenbach

1983, Bunker et al. 1995).

These results suggested that, in the point of gene expressions, the thinned plants also showed the same feature indicating excess source ability as sink-limited plants. Some genes were not related to photosynthesis or vegetative storage proteins but involved in carbohydrate metabolism (beta-amylase, Myo- inositol- 1 phosphate synthase, Endo- 1,4- beta- mannanase, Xyloglucan endotraglucosylase/hydorolase), plant hormone relation (Dormancy/auxin associated family protein), and transcription factor positively regulating ethylene biosynthesis (NAC transcription factor). These genes may have unforeknown important roles in GSD occurrences.

3.4.3. Improved light availability at R5+28d and R5+42d had no significant effect on neither GSD severity nor growth parameters related to source ability

In Exp. 3.2, shade removal at R5+28d or at R5+42d had no significant effect on seed weight and stem dry weight (Table 3.7). This indicated that improved light availability after R5+28d, when leaf yellowing starts, had no significant effect on assimilative ability of soybean plants. If GSD occurrences is not related to enhanced source ability but related other physiological changes caused by improved light availability, there is a possibility that shade removal after R5+28d promotes GSD occurrences. However, shade removal at

R5+28d or at R5+42d also had no significant effect on GSD severity (Table 3.5). These results supported the hypothesis that GSD was promoted by enhanced source caused by improved light availability.

3.4.4. Effects of duration of shade removal after R5 on GSD severity corresponded to sink size and enhanced source ability caused by improved light availability

GSD severity became higher as duration of shade removal at R5 became longer (Table 3.5), supporting the hypothesis that GSD was promoted by enhanced source caused by improved light availability. However, treatment 2 showed significantly lower GSD score than control plants (shading from sowing to harvest), which appears to contradict the hypothesis.

Treatment 2 significantly increased seed number per plant compared to control (Table 3.7). And seed number per plant of treatment 2 showed same level as treatments 3 and 4 (Table 3.7). This result corresponded to the previous study showing that sink size was determined by about 10 days after R5 by light environment (Boad & Tan, 1995). On the other hand, only treatment 2 showed significantly lower 100 seeds weight than in control (Table 3.7), which indicated insufficiency of source ability to sink size. These results suggested that improved light availability for 14 days after R5 in treatment 2 increased

sink size as same level as those in treatments 3 and 4 but source ability was not sufficient in treatment 2 compared to treatments 3 and 4 due to shading after R5+14d. As a result, treatment 2 showed insufficiency of source to sink compared to control. Thus, the results of treatment 2 could be also interpreted to support the hypothesis that sink-source balance affects GSD.

3.4.5 Shading treatment itself had an effect to promote GSD occurrences

It was indicated that shading by 82% shade sheet from sowing to harvest promoted GSD occurrences compared to the plots without shade sheet from sowing to harvest (Table 3.6). In chapter 2, 94% shade sheet showed the similar effect to promote GSD occurrences, but 84% shade sheet did not (Table 2.6). In this study, the reason of the phenomenon could not be elucidated. There may be some environmental factors that affects GSD of continuously and severely shaded plants.

3.4.6 Conclusions

In this chapter, it was indicated that the thinned plats showed the feature of excess source ability which was also reported in depodded plants and male-sterile plants in the previous studies (Brown & Hudson, 2017, Bunker et al., 1995, Heitholt & Egli, 1985, Staswic,

1988, Wittenbach 1983) in the both points of dry weight growth and gene expression. It also was indicated that degrees of enhanced source ability and sink size affected by duration or timing of shade removal corresponded to extent of GSD severity. These two facts suggest that GSD occurrences promoted by improved light availability were caused by enhanced source ability without reduced sink. Thus, the sink-source balance hypothesis on the physiological mechanism of GSD could be strongly supported by the studies in chapter 2 and chapter 3.

Chapter 4

Effect of thinning on cultivar differences of green stem disorder in soybean

4.1 Introduction

It has been reported that there are differences in GSD occurrences between cultivars (Pierce et al., 1984; Matsumoto et al., 1986; Furuya & Umezaki, 1993; Mochizuki et al., 2005; Hill et al., 2006; Yamada et al., 2014; Fujii et al. 2015; Isobe et al., 2015). Thus, breeding GSD-insensitive cultivars is expected to be an effective solution to GSD.

During breeding, comparisons of the GSD severity between many cultivars or lines are needed. However, it is difficult to accurately detect the differences in GSD severity between cultivars or lines because the occurrences of GSD largely vary by field location and cultivation year (Fujii et al., 2015; Hill et al., 2006). In particular, it is generally difficult for breeders to find differences in potential GSD sensitivity between cultivars or lines when GSD occurrences are somehow suppressed.

Thus, it is desirable to establish an effective experimental treatment that promotes GSD occurrences in order to stably detect the differences between cultivars or lines. Depodding,

which has often been used in previous studies, promotes the delayed senescence of leaves and stems (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982). However, depodding is a time- and labor-consuming method and is difficult to do, especially in a field experiment. Depodding also has a problem as an experimental technique, because cutting organs induces the expression of stress-response genes and metabolic shifts in distant leaves, which may lead to unintended consequences as well as sink limitation (Turner et al. 2012).

In chapter 2 and chapter 3, it was found that thinning at the R5 growth stage, which can be performed more easily than depodding at the field scale, promoted GSD occurrences in soybean. However, in the previous study, the cultivar used in the experiment was only ‘Sachiyutaka’, the leading cultivar in the region including the experimental field.

Thus, the effectiveness of the thinning treatment as the experimental technique for breeding needs to be validated by confirming (1) whether thinning promotes the GSD occurrences of other cultivars in addition to ‘Sachiyutaka’ and (2) whether differences in GSD severity between cultivars are clarified by thinning.

To confirm these, in this chapter, the thinning treatment was applied on four cultivars, which were evaluated in terms of their GSD severity in an independent study by another

group (Saruta et al. 2012), and the GSD severity and agronomical traits were compared to draw genotypically different characteristics.

4.2 Materials and methods

4.2.1 Plant materials and experimental site

Two experiments were conducted in the experimental fields of NARO, Western Region Agricultural Research Center, Hiroshima, Japan (lat. 34° 30' N, long. 133° 23' E, and 2 m elevation; Typic Fluvaquents soil type). Exp. 4.1 was conducted in 2015. Exp. 4.2 was conducted in 2016 and in 2017. In the experimental fields, the ground water level was maintained at 30 cm below the ground surface by the farm-oriented enhanced aquatic system FOEAS (Wakasugi & Fujimori, 2009). Inorganic fertilizers were applied at 3 g m⁻² of N, 10 g m⁻² of P₂O₅, and 10 g m⁻² of K₂O a day before sowing. Insecticides and fungicides were applied properly to avoid biotic stresses. In 2016, three cultivars, namely, 'Tachinagaha' (Maturity group 5, Ude et al. 2003), 'Sachiyutaka' (Maturity group 6, Fatichin et al. 2013), and 'Hatsusayaka' (equivalent to Maturity group 5) were used (Table 1). In 2017, four cultivars, namely, 'Tachinagaha', 'Tamahomare' (Maturity group 6, Fatichin et al. 2013), 'Sachiyutaka', and 'Hatsusayaka' were used (Table 1). In these

cultivars, the previously judged GSD score (on a 6-point scale) was reported by Saruta et al. (2012) (Table 4.1).

Table 4.1. Dates of growth stage and treatments.

Year	Cultivar	Previously evaluated		The date of the growth stage or treatment				
		GSD score ^a	Treatment ^b	Sowing	R1	R5	Thinning	R8
2016	Tachinagaha	4.8	Dense	27 June	30 July	18 August	-	27 October
			Thinning	27 June	30 July	19 August	26 August	28 October
	Sachiyutaka	2.0	Dense	27 June	4 August	29 August	-	1 November
			Thinning	27 June	4 August	28 August	5 September	1 November
	Hatsusayaka	0.7	Dense	27 June	5 August	3 September	-	2 November
			Thinning	27 June	5 August	3 September	10 September	3 November
2017	Tachinagaha	4.8	Dense	22 June	28 July	20 August	-	1 November
			Thinning	22 June	28 July	21 August	27 August	5 November
			Sparse	22 June	29 July	18 August	-	31 October
	Tamahomare	2.4	Dense	22 June	30 July	25 August	-	5 November
			Thinning	22 June	30 July	26 August	1 September	8 November
			Sparse	22 June	31 July	25 August	-	5 November
	Sachiyutaka	2.0	Dense	22 June	3 August	28 August	-	1 November
			Thinning	22 June	3 August	28 August	4 September	1 November
			Sparse	22 June	1 August	24 August	-	1 November
	Hatsusayaka	0.7	Dense	22 June	3 August	30 August	-	2 November
			Thinning	22 June	4 August	30 August	6 September	3 November
			Sparse	22 June	3 August	29 August	-	1 November

Notes: ^a Previously evaluated GSD score means GSD score evaluated by 6-grade system (0 to 5) in Saruta et al. (2012). ^b Dense, plant population density was kept at 22.2 plants m⁻². Thinning, thinning was conducted at R5. Sparse, plant population density was kept at 5.56 plants m⁻².

4.2.2 Treatments

Exp.4.1: The effect of the timing of thinning on GSD in ‘Sachiyutaka’

The sowing date was 24 June 2015. There were 12 plots (six treatments and two replications). The size of each plot was 3.0 m × 3.3 m. The planting density was either dense (22.2 plants m⁻², 0.3 m row and 0.15 m plant spacing) or sparse (5.56 plants m⁻², 0.6 m row and 0.3 m plant spacing). There were also plots in which thinning treatments were conducted at R1, R1 +14d, R5, and R5 +14d (7 August, 21 August, 4 September, and 18 September). In the thinning treatment, the planting density changed from dense (22.2 plants m⁻²) to sparse (5.56 plants m⁻²) by performing thinning activities. Thinning involved cutting off all the above-ground parts of the plant in every other row of the plot and every other plant in the remaining rows. All data were recorded by sampling individual plants randomly selected from each plot, excluding plants on the border of the plot. The number of plants selected was six. The value for each plot was the average score of the recorded plants, and the mean of the replications was the representative score for each treatment group.

Exp. 4.2: The effect of thinning on cultivar differences in GSD

The sowing dates were 27 June 2016 and 22 June 2017 (Table 4.1). There were 12 plots

(three cultivars, two treatments, and two replications) in 2016 and 24 plots (four cultivars, three treatments, and two replications) in 2017. The size of each plot was 3.0 m × 2.1 m in both years. There were three treatments that differed in their planting density: dense, sparse, and thinning. In the dense and sparse treatments, the planting density was maintained at 22.2 plants m⁻² and 5.56 plants m⁻², respectively, from sowing until R8. In the thinning treatment, thinning was conducted at 6 or 7 days after R5 for each cultivar (Table 4.1). All data were recorded by sampling individual plants randomly selected from each plot, excluding the plants on the border of the plot. The number of plants selected was six in 2016 and nine in 2017. The value for each plot was the average score of the recorded plants, and the mean of the replications was the representative score for each treatment group.

4.2.3 Measurements

The dates of growth stages R1, R5, and R8 were recorded for each plant, following the method of Fehr & Caviness (1977). The severity of GSD was assessed for each plant at the R8 stage using a scoring method slightly modified from that of Furuya & Umezaki (1993) (chapter 2). In the scoring method, the GSD score (1-5) was assigned based on the stem color and number of leaves left on the stem at R8. A high GSD score represents

severe GSD symptoms. In Exp.4.2, seed weight was measured for each plant sampled after R8 stage. Pod number per node were calculated by the number of pods and the number of total nodes counted for each plant sampled after R8 stage. The dry matter N concentration of the main stem was measured using a Vario MAX CN (Elementar Analysensysteme GmbH, Germany). Three nodes at the center of the main stem were sampled and analyzed. In Exp. 4.2, several plants were randomly selected from each plot in the plants thinned after R5, excluding the plants on the border of the plot, except ‘Tamahomare’ in 2017, in order to investigate cultivar differences of plants in dense plant population at R5 growth stage in leaf area, the number of leaves, dry weight of leaflets, and the dry matter N concentration of the main stem. The number of plants selected was six in 2016 and nine in 2017. Leaf area was measured using a LI-3100C (LI-COR). Dry weight of leaflets was measured after drying at 80°C for 3 days.

4.2.4 Statistical analysis

Experiments were conducted in a completely randomized design. Analysis of variance (ANOVA) and Tukey’s test or t-test were used to test the differences in values and compare the means between the treatment groups or cultivars ($p < 0.05$ or $p < 0.01$). GSD scores were analyzed after Box-Cox transformation. The correlation coefficient of the

GSD score and the N concentration of the main stem for each plant sampled were calculated. All analyses were performed using the statistical software BellCurve for Excel (Social Survey Research Information Co., Ltd.).

4.3 Results

4.3.1 Effect of the growth stage of the thinning treatment on the severity of green stem disorder

The GSD scores of the treatment of thinning at R5 (3.7) and R5+14d (3.5) were significantly higher than those of the treatment kept dense or the sparse plant population (2.3) (Table 4.2). The GSD scores of the thinning treatments at R1 and R1+14d were not significantly different from those of the dense or sparse treatments or from the thinning treatments at R5 and R5+14d, showing intermediate values (Table 4.2). There was no difference in GSD score between the treatments with thinning at R5 and thinning at R5+14d (Table 4.2).

Table 4.2. The effects of the timing of thinning on GSD in 'Sachiyutaka' .

Cultivar	Treatment	The growth stage of thinning	GSD score
Sachiyutaka	Thinning	Dense	2.3b
		R1	2.8ab
		R1+14d	3.1ab
		R5	3.7a
		R5+14d	3.5a
	Sparse	-	2.3b
ANOVA			*

Notes: Within columns, means followed by the same letter are not significantly different according to Tukey's test (0.05).

*Significant at the 0.05 probability level. The GSD scores of Dense, thinning at R1, thinning at R5, and Sparse treatment were the data used in Yamazaki et al. (2018).

4.3.2 Differences in severity of green stem disorder between treatments in each cultivar and between cultivars in each treatment

In the average of the two years, the GSD scores of the thinning treatment were significantly higher than those of the dense treatment in both 'Sachiyutaka' and 'Hatsusayaka' (Table 4.3). Meanwhile, in 'Tachinagaha', there were no significant differences in GSD scores between the thinning treatment and the dense treatment.

In the comparison between the three planting density treatments in 2017 (Table 4.4), there were no significant differences in GSD score between the dense treatment and the sparse

treatment within each cultivar. The thinning treatment, however, showed significantly higher GSD scores than the other treatments in all cultivars except ‘Tachinagaha’.

In the dense treatment, the GSD score of ‘Tachinagaha’ (4.0) was significantly higher than those of the other two cultivars (Table 4.3). There were no significant differences between ‘Sachiyutaka’ (2.1) and ‘Hatsusayaka’ (1.8) in terms of their GSD score.

In the thinning treatment, ‘Tachinagaha’ showed the highest GSD score (4.3); the next was ‘Sachiyutaka’ (3.6), and ‘Hatsusayaka’ showed the lowest (2.8) (Table 4.3). This order is the same as the order of the previously evaluated GSD scores (Table 4.1).

In the results of three-way ANOVA (Table 4.3), there were no significant differences by year, while there were significant differences in the cultivars, treatments, and interactions of cultivar and treatment.

Table 4.3. The effects of thinning on GSD, growth duration, N concentration, and sink size (mean of two-year experiment).

Cultivar	Treatment ^a	GSD score ^b	R8 (DAS)	N (mg g ⁻¹) ^c	Seed weight (g plant ⁻¹)	Pod number per node
Tachinagaha	Dense	4.0ab	127	5.8bc	17.8b	1.25bc
	Thinning	4.3a	130	9.8a	22.0b	1.75a
Sachiyutaka	Dense	2.1cd	130	3.8c	20.7b	1.19c
	Thinning	3.6b	130	9.5ab	32.7a	1.63ab
Hatsusayaka	Dense	1.8d	131	3.0c	20.4b	1.18c
	Thinning	2.8c	132	3.8c	26.9b	1.47abc
Year		ns	**	ns	ns	**
Cultivar		**	ns	**	*	ns
Treatment		**	ns	**	**	**
ANOVA	Year*Cultivar	ns	*	ns	ns	ns
	Year*Treatment	ns	ns	ns	ns	ns
	Cultivar*Treatment	**	ns	*	ns	ns
	Year*Cultivar*Treatment	ns	ns	ns	ns	ns

Notes: Within columns, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**Significant at the 0.01 probability level; *Significant at the 0.05 probability level. ns, nonsignificant at the 0.05

probability level.^aDense, plant population density was kept at 22.2 plants m⁻². Thinning, the thinning treatment was conducted at R5.

^bANOVA and Tukey's test were conducted after Box-Cox transformation. A part of the data in Yamazaki et al. (2018) were used as the GSD scores of 'Sachiyutaka'.

^cN (mg g⁻¹) is dry matter N concentration in main stem.

Table 4.4. The effect of thinning on GSD in Exp. 4.2 in 2017.

Cultivar	Treatment ^a	GSD score
Tachinagaha	Dense	4.0a
	Thinning	4.2a
	Sparse	4.2a
Tamahomare	Dense	2.1cd
	Thinning	3.9a
	Sparse	2.3cd
Sachiyutaka	Dense	1.8d
	Thinning	3.6ab
	Sparse	2.0cd
Hatsusayaka	Dense	1.7d
	Thinning	3.0bc
	Sparse	1.6d
ANOVA	Cultivar	**
	Treatment	**
	Cultivar*Treatment	**

Notes: Within columns, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**Significant at the 0.01 probability level.

^aDense, plant population density was kept at 22.2 plants m⁻².

Thinning, the thinning treatment was conducted at R5. Sparse, plant population density was kept at 5.56 plants m⁻².

4.3.3 Cultivar differences in seed weight, number of pods, and leaf growth at R5

There were no significant differences between cultivars in terms of the seed weight per plant and the number of pods per node in the dense treatment (Table 4.3). In the thinning treatment, the seed weight per plant of 'Sachiyutaka' was significantly larger than any those of the other cultivars, and the number of pods per node did not differ between cultivars (Table 4.3). The seed weight per plant in the thinning treatment was

significantly higher than that in the dense treatment only in ‘Sachiyutaka’, although the seed weight per plant tended to increase in the thinning treatment compared to the dense treatment in the other two cultivars (Table 4.3). The increasing rates of seed weight per plant by thinning were 23.6 % in ‘Tachinagaha’ (22.0 g plant⁻¹ to 17.8 g plant⁻¹), 58.0 % in ‘Sachiyutaka’ (32.7 g plant⁻¹ to 20.7 g plant⁻¹), and 31.9 % in ‘Hatsusayaka’ (26.9 g plant⁻¹ to 20.4 g plant⁻¹) (Table 4.3). The numbers of pods per node were significantly higher in the thinning treatment than in the dense treatment in ‘Tachinagaha’ and ‘Sachiyutaka’ (Table 4.3).

4.3.4 N concentration of the main stem

The N concentrations of the main stem sampled at R5 stage in the dense plant population were not different between the three cultivars or between two years (Table 4.5). The N concentration in the main stem sampled after the R8 stage in ‘Tachinagaha’ and ‘Sachiyutaka’ with thinning was significantly higher than that in the dense treatment (Table 4.3). The dense treatment of ‘Tachinagaha’ also tended to have higher N concentrations when compared with ‘Hatsusayaka’ (Table 4.3). These three groups which showed high N concentration in the main stem corresponded to the groups which showed significantly high GSD scores (Table 4.3).

The regression lines of the correlation between the N concentration in the main stem and GSD score are shown in Fig. 4.1. ‘Sachiyutaka’ in Exp. 4.1 and in Exp. 4.2 and ‘Hatsusayaka’ showed significant coefficients of determination (R^2) (Fig. 4.1). The inclinations of the regression lines were smaller in ‘Tachinagaha’ (0.048) and larger in ‘Hatsusayaka’ (0.671) compared to those in ‘Sachiyutaka’ in Exp. 4.2 (0.196) and Exp. 4.1 (0.100) (Fig. 4.1).

Table 4.5. Leaf growth at R5 of each cultivar (mean of two-year experiments).

Cultivar	Leaf area (cm ² plant ⁻¹)	Leaf number (plant ⁻¹)	Dry weight of		N (mg g ⁻¹) ^a
			leaflets (g plant ⁻¹)		
Tachinagaha	2463	15.8	9.2		11.5
Sachiyutaka	2724	18.1	9.7		11.9
Hatsusayaka	2526	17.0	9.4		11.8
ANOVA	Cultivar	ns	ns	ns	ns
	Year	ns	ns	ns	ns
	Interaction	ns	ns	ns	ns

Note: ns, nonsignificant at the 0.05 probability level.

^aN (mg g⁻¹) is dry matter N concentration in main stem.

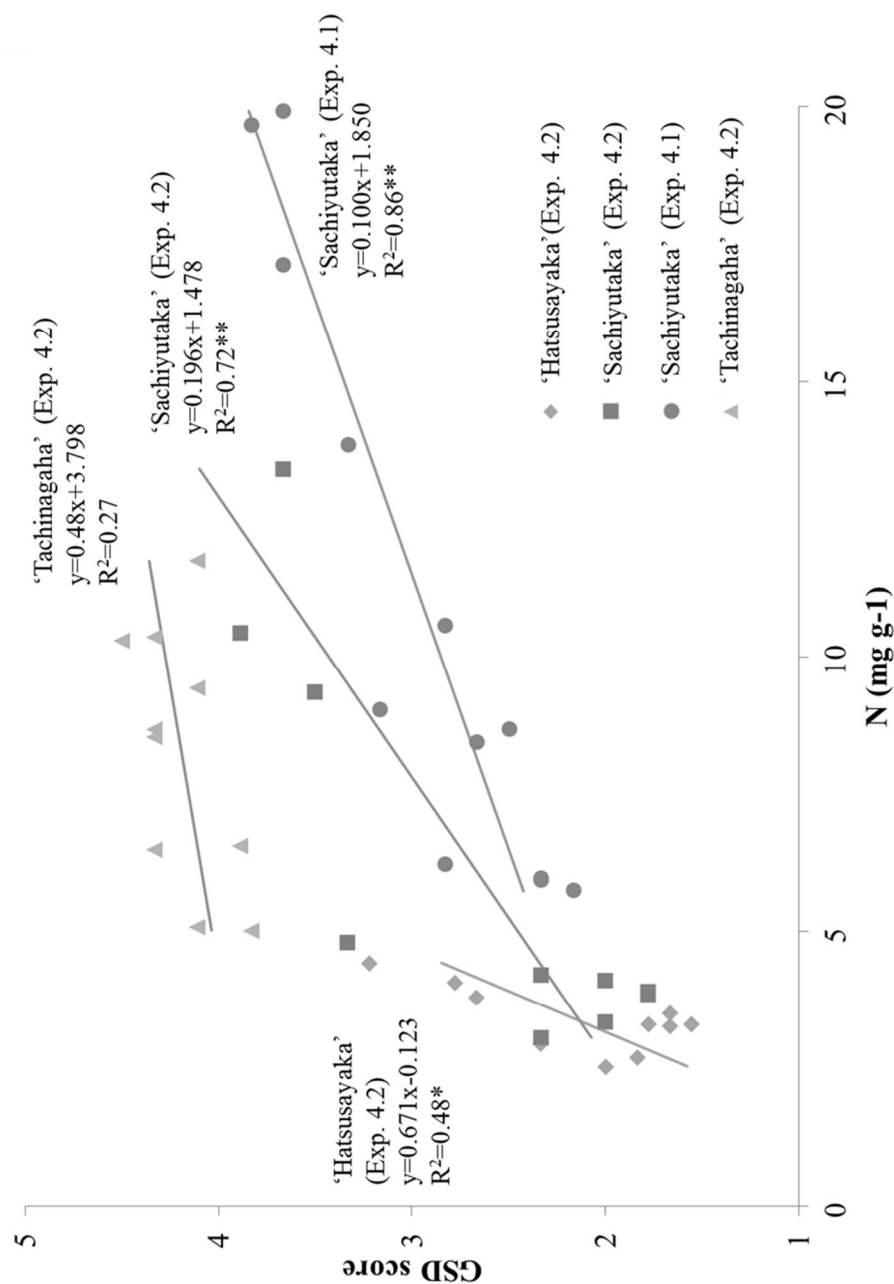


Fig. 4.1. The correlation between N concentration in the main stem and GSD scores of 'Sachiyutaka' in Exp. 4.1 (n=12), 'Tachinagaha' (n=10), 'Sachiyutaka' (n=10), and 'Hatsusayaka' (n=10) in two-year experiments in Exp. 4.2.

Notes: Each point in the figure indicates the average score of 6 or 9 plants in a replication.

4.4 Discussion

4.1 Thinning generally promotes GSD occurrences in soybean cultivars

In chapter 2, thinning did not affect the developmental progression of the reproductive stage, which was evaluated based on pod maturation, suggesting that GSD promoted by thinning was characterized by the delayed maturation of the leaves and stems. In the present results, the days from sowing to R8 were also not significantly different between treatments and between cultivars (Table 4.3), suggesting that the GSD occurrences promoted by thinning or cultivar differences in our experiments were characterized by the delayed maturation of the leaves and stems.

As a result of the two-year experiment, not only ‘Sachiyutaka’ but also ‘Hatsusayaka’ showed significantly severe GSD symptoms in the thinning treatment compared to those in the dense treatment (Table 4.3). In ‘Tamahomare’, the thinning treatment showed severe GSD symptoms compared to the dense or sparse treatment, which were the same as those shown by ‘Sachiyutaka’ and ‘Hatsusayaka’, although that was the result of only one year (Table 4.4). These results suggest that the phenomenon that thinning promotes GSD occurrences is not limited to ‘Sachiyutaka’ but is seen generally in soybean cultivars. However, ‘Tachinagaha’ without thinning treatment showed significantly severe GSD

symptoms compared to the other cultivars, and there were no differences between treatments (Table 4.3). This fact suggests that in ‘Tachinagaha’, there was another factor unrelated to the thinning treatment that promotes GSD occurrences, due to which the effect of thinning on GSD severity could not be detected as statistically significant differences.

4.2. Thinning clarified differences in GSD severity between cultivars

In Exp. 4.2, there was no significant difference in GSD severity without thinning between ‘Sachiyutaka’ and ‘Hatsusayaka’ (Table 4.3). On the other hand, in the thinning treatment group, there was a significant difference between ‘Sachiyutaka’ and ‘Hatsusayaka’. In addition, the order of GSD severity of cultivars corresponded to the order of the previously judged GSD severity (Saruta et al., 2012). These facts indicated that thinning treatment magnifies cultivar differences that could not be easily detected without thinning treatment. Thus, although more cultivars need to be tested to prove, the thinning treatment could be used as a useful experimental technique to stably detect cultivar differences in breeding, especially when GSD occurrences are generally scarce and there are little differences between cultivars.

4.3 The timing of thinning and cultivar differences in GSD

In Exp. 4.2, the timing of thinning differed between cultivars by 15 days in 2016 and 10 days in 2017 because the date of the R5 growth stage differed between cultivars (Table 1). Thus, we cannot deny the possibility that the differences in GSD scores between cultivars in the thinning treatment at R5 were caused by the different timing of thinning, which possibly lead to unequal meteorological conditions. However, in Exp. 4.1, both thinning at R5 and thinning at R5+14d significantly promoted GSD occurrences compared to the dense or sparse groups, and there was no significant difference in GSD occurrences between the former two treatments (Table 4.2). These results suggest that the effect of thinning on GSD severity does not significantly change, at least during the 14 days after R5. Although there were significant differences in GSD score between ‘Sachiyutaka’ and ‘Hatsusayaka’ subjected to the thinning treatment, the differences in the timing of thinning were only 5 days in 2016 and 2 days in 2017 (Table 4.1). This result suggests that the differences in GSD score between ‘Sachiyutaka’ and ‘Hatsusayaka’ subjected to the thinning treatment were not caused by the timing of the thinning treatment but rather by some genotypic factors.

4.4 Sink and source size and the cultivar differences in GSD

As the mechanism of GSD occurrences has been suggested to be related to sink-source balance (Egli & Bruening, 2006; Wittenbach, 1983a, 1983b), cultivar differences in GSD may be caused by differences in sink-source balance, although there has been no study of the relation between the cultivar differences of GSD and sink-source balance. In other words, it could be that cultivars that have a smaller sink size or a larger source ability may show higher GSD scores.

However, in the present study, there were no significant differences in the sink size between cultivars in the dense treatment group, which was shown as the seed weight per plant or the number of pods per node, although there were significant differences in GSD score between cultivars (Table 4.3). In the thinning treatment group, ‘Sachiyutaka’ had significantly larger seed weight per plant than ‘Hatsusayaka’, although ‘Sachiyutaka’ showed significantly higher GSD scores than ‘Hatsusayaka’ (Table 4.3). These results suggested that different sink size itself was not the cause of differences in GSD severity among cultivars. The results agreed with the reports of Isobe et al. (2015), which demonstrated no correlation between growth parameters and GSD severity in several cultivars.

Meanwhile, the size of photosynthetically assimilative organs (leaf area, the number of leaves, and dry matter of leaflets) at R5, which partly contributes to source ability (Saeki.

1960), showed no difference between cultivars (Table 4.5). Thus, it should be examined whether leaf photosynthetic ability, which is also related to source ability (Long et al., 2006; Zhu et al., 2010), as well as the nutrient absorption ability of root and assimilate translocation activities, are the cause of the cultivar differences in GSD.

From the viewpoint of leaf photosynthetic ability, the result that the increasing ratio of seed weight per plant by the thinning treatment in ‘Sachiyutaka’ tended to be higher than that of ‘Hatsusayaka’ is also notable (Table 4.3). Given that the increasing ratio of seed weight per plant indicated enhanced photosynthetic ability by light enrichment caused by thinning (Schou et al., 1976), the positive effect of thinning on photosynthetic ability may be larger in ‘Sachiyutaka’ than in ‘Hatsusayaka’. This may have caused the thinning treatment to magnify the differences in GSD severity between ‘Sachiyutaka’ and ‘Hatsusayaka’.

4.5 Cultivar differences in the correlation between the N concentration in the main stem and GSD severity

In chapter 2, the N concentration in the main stem at maturity was significantly increased by the thinning treatment at R5, and it was positively and significantly correlated to the GSD score in ‘Sachiyutaka’. This result corresponded to the report that depodded GSD

soybean plants tend to have high dry matter N concentration in the stem compared to controls (Egli & Bruening, 2006).

In the present study, the N concentrations in the main stem also showed the same tendency in each cultivar in Exp. 4.1 and in Exp. 4.2. Especially, there were statistically significant positive correlations in ‘Sachiyutaka’ in both Exp. 4.1 and Exp. 4.2 and ‘Hatsusayaka’ (Fig. 4.1), and the thinning treatment significantly increased the N concentrations in the main stem in ‘Tachinagaha and ‘Sachiyutaka’ in Exp. 4.2 (Table 4.3).

In the comparisons of cultivars in the same treatment in Exp. 4.2, the N concentration in the main stem increased as the GSD score increased (Table 4.3). In addition, there were no differences between cultivars in terms of the N concentration in the main stem at the R5 growth stage, and the values at R5 (11.5 mg g⁻¹ to 11.9 mg g⁻¹) were higher than the values at R8 (3.0 mg g⁻¹ to 9.8 mg g⁻¹) in each cultivar, regardless of the thinning treatment (Table 4.3, Table 4.5). These results suggested that a positive correlation between GSD severity and N concentration in the main stem can generally be seen in soybean cultivars, and differences in N concentration in the main stem were characterized by decreased N concentrations from the R5 growth stage to the R8 growth stage.

However, the increasing rate of GSD score with an increase in N concentration in the

main stem indicated by the inclination of the regression line in Fig. 4.1 differed between cultivars. The increasing rate of the GSD score with N concentration in the main stem was smaller in 'Tachinagaha' than in 'Sachiyutaka' and was larger in 'Hatsusayaka' than in 'Sachiyutaka'. These results imply that the severity of the GSD response to N concentrations in the main stem differed between cultivars. Depodded soybean showed expression of vegetative storage proteins in vegetative organs, suggesting that a surplus of assimilation products accumulate (Wittenbach 1983a; Wittenbach 1983b; Ogiwara & Ishikura 1994) and lead to high N concentrations in the main stem in vegetative organs in GSD plants. Thus, the maturation of the leaf and stem is seemingly delayed with the surplus accumulation in 'Sachiyutaka', while the surplus might be critical to the delay of maturation in 'Hatsusayaka'. In 'Tachinagaha', the delay of maturation was induced regardless of increasing N concentration in the main stem by thinning treatment. This may indicate that there is another factor to induce the delay which is not related to the N surplus in 'Tachinagaha'. Zhao et al. (2014) reported that there were cultivar differences in nitrogen redistribution and its contribution to seed yield. This cultivar differences may be related to the differences in relationships between N surplus and GSD severity.

There was a slight difference in the inclination of the regression line between Exp. 4.1 and Exp. 4.2 in 'Sachiyutaka', suggesting that some environmental factors influence the

severity of the GSD response to N surplus.

Chapter 5

General Discussion

5.1 Thinning at R5 growth stage as a new experimental method to promote GSD occurrences

In this study, it was indicated that improved light availability by thinning and shade removal promoted GSD occurrences. Particularly, thinning is a simpler, less time- and labor- consuming method than depodding, which has been used in many studies of GSD. In fact, all works for the thinning treatment could be completed by myself alone in a single day at every treatment in this study.

In addition to this, thinning is a stable method to promote GSD occurrences because thinning significantly promoted GSD severity compared to dense treatment in every 5-year in this study. In the viewpoint of cultivar differences, thinning is also a stable method because thinning significantly promoted GSD severity of 3 different cultivars ('Tamahomare', 'Sachiyutaka', and 'Hatsusayaka') in this study. The result suggested that GSD promoted by thinning can be generally applied regardless of cultivar differences

although a cultivar like ‘Tachinagaha’, which was promoted GSD occurrences by another factor unrelated to the thinning treatment, had no significant differences among treatments. However, the experimental treatment to promote GSD occurrences was needed in the situation when the level of GSD occurrences is low without any treatment. Thus, it was not a problem for the effectiveness of the thinning treatment as an experimental method to study GSD.

Depodding has a problem as an experimental method to study GSD in the point that injury stresses by cutting organs may cause unintended physiological effects on soybean plants. Injury stresses by cutting organs and reduced sink cannot be separated in the depodding experiments. In some studies, it was attempted to solve this problem by using male-sterile soybean (Turner et al. 2012). On the contrary, cutting organs is not involved in the thinning treatment. Thus, injury stresses by cutting organs and enhanced source can be discussed separately in the thinning treatment

5.2 Experiments of enhanced source ability by thinning or shade removal supported sink-source balance hypothesis

In the previous studies, only the effect of reduced sink by depodding or several stresses

on GSD occurrences was discussed as evidences of the sink-source balance hypothesis. Thus, the possibility that any other possible effect other than the sink-source balance, such as reduced pods itself, has not been denied as the cause of GSD. The ‘Death hormone’ hypothesis, in which some sort of substances transported from the pods to vegetative organs promoted senescence (Engvild, 2006), also may explain this phenomenon, because reduced pods cause reduction of transport of substances from the pods which promote senescence.

In this study, it was suggested that enhanced source capacity without reduced sink also promoted GSD occurrences. This result supported sink-source balance hypothesis.

Mochizuki et al. (2005) reported that GSD plants tended to show a larger above-ground dry weight and seed weight than normally matured plants in the experiment of six soybean cultivars grown in the Temperature Gradient Chambers. This result cannot be explained by reduced sink reported in the previous study. On the contrary, the enhanced source without reduced sink in the present study may explain this phenomenon. Like this case, the present results are expected to be useful for applying the sink-source balance hypothesis to GSD occurrences without reduced pods.

5.3 Subject for further study

There is no quantitative analysis of enhanced source by improved light availability in the thinning treatment and the shade removal treatment in this study. In other words, there is no data of how photosynthesis of individual plant increased by thinning or shade removal. In order to quantify enhanced source, photosynthetic rate of each leaf of different position in a plant from R5 to R8 needs to be analyzed and compared between thinned or shade removed plants and control plants. If this data could be obtained, sink-source balance may be quantified by sink/source ratio and mathematical model which determine GSD severity may be developed.

In this study, the role of roots in GSD occurrences was not considered. In order to quantify the amount of photosynthesis, dry matter of roots also needs to be analyzed. Isobe et al. (2015) indicated that higher cytokinin level in xylem exudate corresponded to more severe GSD symptom. The amount of cytokinin synthesized in roots needs to be analyzed and compared in the thinned or shade removed plants and control plants.

GSD score was used to evaluate GSD severity in this study. GSD score has been used in many studies (Furuya & Umezaki, 1993; Fujii et al. 2015; Yamada et al., 2014; Isobe et al., 2015; Mochizuki et al., 2005) but it involves subjective evaluation of stem color. In this study, quantitative parameters which indicate GSD severity, namely, the number of

leaves left at R8 growth stage and N concentration in main stem corresponded to GSD score. Thus, the conclusions in this study was supported by objective evaluation of GSD severity. However, the method to quantify GSD severity which could be conducted easily is needed in the future study.

In chapter 2 and chapter 3, the phenomenon that continuous shading from sowing to harvest by the shade equipment promoted GSD occurrences compared to the treatment with no shade-sheet from sowing to harvest could be seen. This phenomenon occurred by the 94% shade-sheet in 2015. However, in 2016, 2017, and 2018, continuous shading by the 82% shade-sheet also promoted GSD occurrences. In this study, the cause of this phenomenon cannot be explained. Further studies are needed to solve this problem.

In chapter 4, the possibility of applying the thinning treatment to breeding GSD insensitive cultivars was indicated. In order to prove this possibility, more cultivars need to be tested and GSD insensitive lines or QTLs must be actually detected. The thinning method may leave possibility of simplification. For example, area of one plot may be able to be smaller than the plot of this study.

5.4 Conclusions

Firstly, the thinning treatment at R5 growth stage promoted GSD occurrences. The experiment of the shade removal treatment showed that this effect was caused by improved light availability (Chapter 2).

Secondly, the sink-source balance hypothesis was verified by using the thinning treatment and the shade removal treatment. The experiment of varied duration and timing of shade removal suggested that GSD severity corresponded to the degree to which source capacity was enhanced by improved light availability. The plants with thinning treatment showed same pattern in growth parameters and gene expression as the depodded plants in the previous studies. This pattern indicated excess the source relative to the sink size. Thus, these results supported the sink-source balance hypothesis (Chapter 3).

Thirdly, the possibility to apply the thinning treatment on breeding GSD insensitive cultivars was verified. The thinning treatment promoted GSD severity of several soybean cultivars. The difference between cultivars in GSD severity became more evident than without thinning treatment. Thus, the possibility that the thinning treatment could be useful as a phynotyping technique in the breeding of GSD insensitive cultivars (Chapter 3).

In this study, it was indicated that the thinning treatment could be the effective method to easily and stably generate GSD for study of GSD in both physiological mechanism and

breeding. The thinning treatment is expected to be used in GSD study in the future.

Acknowledgements

At completing my three years' work at the Laboratory of Crop Science, Graduate School of Agriculture, Kyoto University, I would like to acknowledge the following people for assisting my research. I could never complete my work without their assistances.

Above all, I would like to express my deepest appreciation to Professor Tatsuhiko Shiraiwa for his continuous direction, support, and suggestion throughout this study. I also would like to express my deepest gratitude to Dr. Tomoyuki Katsube-Tanaka for his continuous support and suggestion throughout this study. I am grateful to Dr. Yu Tanaka for his insightful advice.

I would like to express my gratitude to Dr. Hideyuki Funatsuki (NARO), Dr. Masahiro Kamei (NARO), Dr. Akinori Okabe (NARO), Dr. Katsuyuki Katayama (NARO), Dr. Hideo Hamaguchi (NARO), Dr. Hiroyuki Takeda (NARO), and Dr. Yohei Kawasaki (NARO) for their insightful advice to the study and support for field experiments.

I am greatly appreciating to Dr. Eri Ogiso-Tanaka (NARO) for transcriptome analysis in chapter 3. Analyzing N concentration of stems in chapter 2 and chapter 4 was supported by Dr. Gen Ishioka (NARO). A part of the experiment in chapter 4 was supported by a research grant from the Takano Life Science Research Foundation.

I never forget my special thanks to the members of Technical Support Center Operations Unit-1 and the other staff members of the Western Region Agricultural Research Center, NARO for their technical assistance in conducting the experiments. I also express my special thanks to the members of the Laboratory of Crop Science, Graduate School of Agriculture, Kyoto University for their insightful suggestion to my study.

Finally, I deeply appreciate my parents and brother for their continuous supports and encouragements throughout my work.

January 2019

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References

Board, J.E., & Tan., Q. (1995). Assimilatory capacity effects on soybean yield components and pod number. *Crop Sci.*, 35, 846-851.

Boethel, D.J., Russin, J.S., Wier, A.T., Layton, M.B., Mink, J.S., & Boyd., M.L. (2000). Delayed maturity associated with southern green stink bug (Heteroptera: Pentatomidae) injury at various soybean phenological stages. *J. Econ. Entomol.*, 93, 707-712.

Brown, A.W., & Hudson, K.A. (2017). Transcriptional profiling of mechanically and genetically sink-limited soybeans. *Plant Cell Environ.* 40, 2307-2318.

Bunker, T.W., Koetje, D.S., Stephenson, L.C., Creelman, R.A., Mullet, J.E., & Grimes, H.D. (1995). Sink limitation induces the expression of multiple soybean vegetative lipoxygenase mRNAs while the endogenous jasmonic acid level remains low. *The Plant Cell*, 7, 1319-1331.

Burke, J.J., Kalt-Torres, W., Swafford, J.R., Burton, J.W., & Wilson, R.F. (1984).

Studies on genetic male-sterile soybeans III. The initiation of monocarpic senescence.

Plant Physiol., 75, 1058-1063.

Crafts-Brandner, S.J., Below, F.E., Harper, J.E., & Hageman, R.H. (1984). Effects

of pod removal on metabolism and senescence of nodulating and nonnodulating soybean

isolines. Plant Physiol., 75, 311-317.

Crafts-Brandner, S.J., & Egli, D.B. (1987). Sink removal and leaf senescence in

soybean. Plant Physiol., 85, 662-666.

Egli, D.B. (2010). Soybean reproductive sink size and short-term reductions in

photosynthesis during flowering and pod set. Crop Sci., 50, 1971-1977.

Egli, D. B., & Bruening, W. P. (2006). Depodding causes green-stem syndrome in

soybean. Crop Management. doi:10.1094/CM-2006-0104-01-RS

Engvild, K.C. (1989). The death hormone hypothesis. Physiologia Plantarum. 77, 282-

285.

Fatichin, Zheng, S. H., Narasaki, K., & Arima, S. (2013). Genotypic adaptation of soybean to late sowing in southwestern Japan. *Plant Prod. Sci.*, 16, 123–130.

Fehr, W.R., & Caviness., C.E. (1977). Stages of soybean development. Spec. Rep. 80. Iowa Agric. Home Econ. Exp. Stn. Iowa State Univ., Ames.

Fujii, K., Kato, S., Sayama, T., Tanaka, Y., Nakazaki, T., Ishimoto, M., & T. Shiraiwa. (2015). Stability verification of the effects of stem determination and earliness of flowering on green stem disorder of soybean against genetic background and environment. *Plant Prod. Sci.*, 18, 166-179.

Furuya, T., Matsumoto, S., Shima, M., & Muraki, K. (1988). Maturation process of top organs in delayed stem maturation soybean plant. (In Japanese with English abstract.) *Jpn. J. Crop Sci.*, 57, 1-7.

Furuya, T., & Umezaki, T. (1993). Simplified distinction method of degree of delayed

stem maturation of soybean plants. (In Japanese with English abstract.) Jpn. J. Crop Sci. 62, 126-127.

Harbach, C.J., Allen, T.W., Bowen, C.R., Davis, J.A., Hill, C. B., Leitman, M., Leonard, B. R., Mueller, D. S., Padgett, G.B., Phillips, X. A., Schneider, R. W., Sikora, E. J., Singh, A. K., & Hartman, G.L. (2016). Delayed senescence in soybean: terminology, research update, and survey results from growers. Plant Health Prog., 17, 76-83.

Hayati, R., Egli, D.B., & Crafts-Brandner., S.J. (1995). Carbon and nitrogen supply during seed filling and leaf senescence in soybean. Crop Sci., 35, 1063-1069.

Heitholt, J.J., & Egli, D. (1985) Influence of deflowering on dry matter production of soybeans. Field Crop. Res., 12, 163-173.

Hill, C.B., Bowen, C.R., & Hartman, G.L. (2013). Effect of fungicide application and cultivar on soybean green stem disorder. Plant Dis., 97, 1212-1220.

Hill, C.B., Hartman, G.L., Esgar, R., & Hobbs, H.A. (2006). Field evaluation of green stem disorder in soybean cultivars. *Crop Sci.*, 46, 879-885.

Hobbs, H.A., Hill, C.B., Gran, C.R., Koval, N.C., Wang, Y., Pedersen, W.L., Domier, L.L., & Hartman, G.L. (2006). Green stem disorder of soybean. *Plant Dis.*, 90, 513-518.

Htwe, N.M.P.S., Yuasa, T., Ishibashi, T., Tanigawa, H., Okuda, M., Zheng, S.H., & Iwaya-Inoue, M. (2011). Leaf senescence of soybean at reproductive stage is associated with induction of autophagy-related genes, GmATG8c, GmATG8i and GmATG4. *Plant Prod. Sci.*, 14, 141-147.

Isobe, K., Ozaki, K., Saito, K., Hatoya, D., Higo, M., & Torigoe, Y. (2015). Varietal difference in the occurrence of delayed stem senescence and cytokinin level in the xylem exudate in soybeans. *Plant Prod. Sci.*, 18, 356-364.

Leopold, A.C., Niedergang-Kamien, E., & Janick, J. (1959). Experimental modification of plant senescence. *Plant Physiol.*, 34, 570-573.

Long, S.P., X.G. Zhu, S.L. Naidu, & D.R. Ort. (2006). Can improvement in photosynthesis increase crop yields? *Plant Cell Environ.*, 29, 315–330.

Mason, H.S., Guerrero, F.D., Boyer, J.S., & Mullet, J.E. (1988). Proteins homologous to leaf glycoproteins are abundant in stems of dark-grown soybean seedlings. Analysis of proteins and cDNAs. *Plant Mol. Biol.*, 11, 845-856.

Mason, H.S. & Mullet, J.E. (1990). Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. *Plant Cell.*, 2, 569-579.

Mathew, J.P., Herbert, S.J., Andreas, S.Z., Rautenkranz, A.F., & Litchfield, G.V. (2000). Differential response of soybean yield components to the timing of light enrichment. *Agron. J.*, 92, 1156-1161.

Matsumoto, S., Furuya, T. & Matsunaga, R. (1986). The occurrence of delayed stem maturation in early soybean varieties and a method for visual distinction. *Jpn. J. Crop Sci.* 55: 333-338.

Mochizuki, A., Shiraiwa, T., Nakagawa, H., & Horie, T. (2005). The effect of temperature during the reproductive period on development of reproductive organs and the occurrence of delayed stem senescence in soybean. (In Japanese with English abstract.) Jpn. J. Crop Sci., 74, 339-343.

Mondal, M.H., Brun, W.A., & Brenner, M.L. (1978). Effects of sink removal on photosynthesis and senescence in leaves of soybean (*Glycine max* L.) plants. Plant Physiol., 61, 394-397.

Nagano, A. J., Honjo, M. N., Mihara, M., Sato, M., & Kudoh, H. (2015). Detection of plant viruses in natural environments by using RNA-Seq. Methods Mol. Biol. 1236, 89–98.

Nobuyasu, H., Liu, S., Adu-Gyamfi, J. J., Mohapatra, P. K., & Fujita, K. (2003). Variation in the export of ¹³C and ¹⁵N from soybean leaf: the effects of nitrogen application and sink removal. Plant and Soil., 253, 331-339.

Ogiwara, H. (2002). Chapter 3. Cultivation technique. Section 11. Delayed leaf senescence. (In Japanese.) Agriculture, Forestry and Fisheries Research Council of Japan, ed., Soybean-technical development for improving national food self-sufficiency ratio. Annotated bibliography of agriculture, forestry and fisheries research, No. 27, 291-294.

Ogiwara, H., & Ishikura, N. (1994). Physiological analysis of delayed stem maturation in soybean plants and possibility of its prediction. (In Japanese.) Jpn. J. Crop Sci., 63(Extra issue 2), 201-202.

Phillips, D.A., Pierce, R.O., Edie, S.C., Foster, K.W., & Knowles, P.F. (1984). Delayed leaf senescence in soybean. Crop Sci., 24, 518-522.

Pierce, R.O., Knowles, P.F. & Phillips, D.A. (1984). Inheritance of delayed leaf senescence in soybean. Crop Sci. 24: 515-517.

Rolland, F., Baena-Gonzalez, E., Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu. Rev. Plant. Biol., 57, 675-709.

Saeki, T. (1960). Interrelationships between leaf amount, light distribution and total photosynthesis in a plant community. *Bot. Mag. Tokyo.*, 83, 55-63.

Saruta, M., Takada, Y., Okabe, A., Kikuchi, A., Ono, S., Igita, K., Sakai, S., Matsunaga, R., Hajika, M., Takahashi, M., & Komatshu, K. (2012). A New Soybean Cultivar ‘Hatsusayaka’, with Tolerance of Delayed Leaf Senescence and Suitability for Tofu Processing. *Bull. NARO West. Reg. Agric. Res. Cent.*, No.11, 81-99.

Sato, J., Shiraiwa, T., Sakashita, M., Tsujimoto, Y., & Yoshida, R. (2007). The occurrence of delayed stem senescence in relation to trans-zeatin riboside level in the xylem exudate in soybeans grown under excess-wet and drought soil conditions. *Plant Prod. Sci.*, 10, 460-467.

Schou, J.B., Jeffers, D.L., & Streeter, J.G. (1978). Effects of reflectors, black boards, or shades applied at different stages of plant development on yield of soybeans. *Crop Sci.*, 18, 29-34.

Schwenk, F. W., & Nickell, C. D. (1980). Soybean green stem caused by Bean Pod

Mottle Virus. Plant Dis. 64:863-865.

Staswick, P.E. (1988). Soybean vegetative storage protein structure and gene expression. Plant Physiol., 87, 250-254.

Staswick, P.E. (1989). Developmental regulation and the influence of plant sinks on vegetative storage protein gene expression in soybean leaves. Plant Physiol., 89, 309-315.

Stephenson, R.A. & Wilson, G.L. (1977). Patterns of assimilate distribution in soybean at maturity. 1. The influence of reproductive developmental stage and leaf position. Aust. J. Agric. Res. 28, 203-209.

Takehara, T., Ochi, S., Ohto, Y., Naito, S., Inoue, H., & Miyagawa, H. (2016). Occurrence of delayed leaf senescence of soybean caused by Rhizoctonia aerial blight in Japan. Japan Agricultural Research Quarterly, 50, 201-208.

Tranbarger, T.J., Franceschi, V.R., Hildebrand, D.F., & Grimes, H.D. (1991). The soybean 94-kilodalton vegetative storage protein is a lipxygenase that is localized in

paraveinal mesophyll cell vacuoles. *Plant Cell*, 3, 973-987.

Tsujimoto, Y., Sato, J., Shiraiwa, T., Tanaka, Y., & Horie., T. (2006). Field investigation of environmental factors causing delayed stem senescence in soybean. (In Japanese with English abstract.) *Kinki J. Crop Sci. and Breed.*, 50, 37-43.

Turner, G.W., Cuthbertson, D.J., Voo, S.S., Settles, M.L., Grimes, H.D. & Lange, B.M. (2012). Experimental sink removal induces stress responses, including shifts in amino acid and phenylpropanoid metabolism, in soybean leaves. *Planta*, 235, 939-954.

Ude, G. N., Kenworthy, W. J., Costa, J. M., Cregan, P. B., & Alvernaz, J. (2003). Genetic diversity of soybean cultivars from China, Japan, North America, and North American ancestral lines determined by amplified fragment length polymorphism. *Crop Sci.*, 43, 1858–1867.

Wakasugi, K., & Fujimori. S. (2009). Subsurface Water Level Control System “FOEAS” that promotes the full use of paddy fields. (In Japanese.) *Journal of the Japanese Society of Irrigation, Drainage and Rural Engineering*, 77, 705-708.

Weaver, L.M., & Amasino, R.M. (2001). Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. *Plant Physiol.*, 127, 876-886.

Wittenbach, V.A. (1982). Effect of pod removal on leaf senescence in soybean. *Plant Physiol.*, 70, 1544-1548.

Wittenbach, V.A. (1983a). Effect of pod removal on leaf photosynthesis and soluble protein composition of field-grown soybeans. *Plant Physiol.*, 73, 121-124.

Wittenbach, V.A. (1983b). Purification and characterization of a soybean leaf storage glycoprotein. *Plant Physiol.*, 73, 125-129.

Yamada, T., Shimada, S., Hajika, M., Hirata, K., Takahashi, K., Nagaya, T., Hamaguchi, H., Maekawa, T., Sayama, T., Hayashi, T., Ishimoto, M., & Tanaka, J. (2014). Major QTLs associated with green stem disorder insensitivity of soybean (*Glycine max* (L.) Merr.). *Breeding Science*, 64, 331-338.

Zhao, X., Zheng, S.H. & Arima, S. (2014). Influence of Nitrogen Enrichment during Reproductive Growth Stage on Leaf Nitrogen Accumulation and Seed Yield in Soybean. *Plant Prod. Sci.*, 17, 209-217.

Zhu, X.G., Long, S.P. Long, & Ort, D.R. Ort. (2010). Improving photosynthetic efficiency for greater yield. *Annu. Rev. Plant Biol.*, 61, 235–261.

List of Publications

(Chapter 2)

Yamazaki, R., Katsube-Tanaka, T., & Shiraiwa, T. (2018) Effect of thinning and shade removal on green stem disorder in soybean. *Plant Prod. Sci.*, 21, 83-92.

(Chapter 4)

Yamazaki, R., Katsube-Tanaka, T., Kawasaki, Y., Katayama, K., & Shiraiwa, T. (2019) Effect of thinning on cultivar differences of green stem disorder in soybean. *Plant Prod. Sci.*, doi.org/10.1080/1343943X.2018.1557531.