Chapter

Nitrogen Fixation in Soybean Nodules Affects Seed Protein and Oil Contents: The Suggested Mechanism from the Coordinated Changes of Seed Chemical Compositions and Phosphoenolpyruvate Carboxylase Activity Caused by Different Types of Nitrogen Fertilizer

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Abstract

The contents of seed storage compounds, protein and oil, determine the best use of soybean seeds, namely materials for food processing and oil production. Genetic and environmental factors could affect the chemical compositions of soybean seeds. However, the mechanisms of how the accumulation of these primary seed compounds is regulated are mostly unclear. In this chapter, we describe the different effects of nodulation on the protein and oil contents in soybean seeds and the crucial role of phospho*enol*pyruvate carboxylase (PEPC) in the protein accumulation of soybean seeds. Based on our previous studies on soybean seeds, we introduce five manners deduced; (1) protein accumulation is independent of oil accumulation, (2) nitrogen fixation results in decreasing oil amount per seed and decreased seed oil content, (3) a high pseudo negative correlation between protein and oil contents in seeds is likely to be observed under less nitrogen supply from the soil, (4) nitrogen absorbed from soil during the late growth stage promote seed production, (5) plant-type PEPC, ex. Gmppc2 in soybean could play a role in amino acid biosynthesis for storage protein accumulation in seeds during the late maturation period.

Keywords: carbon metabolism in immature seeds, Gmppc2, plant-type, principal component analysis, role of PEPC, seed yield, slow-release N fertilizer

1. Introduction

Soybean seeds contain about 40% protein, 20% oil, 35% carbohydrate, and 5% minerals on a weight basis [1]. Contents of these compounds vary among cultivars

(CVs) and environments of plant growth. Seeds having higher protein content are preferable for food material, and which is called food bean. On the other hand, those having higher oil content are for vegetable oil production, and which is called oil bean. Germplasm stock of USDA exhibits a protein concentration from no less than 35% to more than 50% with an oil concentration of 7% to 28% [2]. Contents of these storage compounds were negatively correlated with each other, implying competition of the synthesis of these storage compounds during the seed maturation period. For the production of soybean seeds to have better quality, it was necessary to clarify the mechanism of how these storage compounds contents were genetically controlled and affected by environmental conditions.

One of the characteristics of soybean plants is N_2 fixation ability concerted with symbiotic microorganisms located in nodules on roots. Soybean plants supply photosynthate, sucrose, to nodules as the nutrient. Symbiotic microorganisms in nodules utilize sucrose for the source of energy required for N_2 fixation and the carbon (C) skeleton of nitrogenous compounds, ureides (allantoin and allantoic acid) [3]. Ureides are suitable forms for the transportation of nitrogen (N) in the plant body. Soybean plants supply less amount of sucrose to nodules when the soil offers enough amount of N (as a form of nitrate) [4]. It is well known that a high concentration of soil nitrate depresses the formation of nodules and N_2 fixation [3].

We did experiments to clarify two possible factors to affect seed protein content, the supply of N to seeds and C metabolism in immature soybean seeds. Firstly, we describe that N_2 fixation in nodules affects seed protein content among soybean plants having different N_2 fixation activity grown on soils with different types of coated urea slow-release N fertilizers (CUSLNFs) in Section 2. Secondly, we describe the role of a CO_2 fixing enzyme, phospho*enol*pyruvate carboxylase (PEPC), on the protein accumulation in maturing seeds in Section 3. Here we introduce our results in the experiments on plants cultivated in the field of the Faculty of Agriculture, Kobe University, where the soil was granite-based udorthents.

2. Association of nodulation and N_2 fixation activity with the accumulation of protein and oil in soybean seeds

2.1 Soybean plants with different nodulation status reveals the effect of nodulation and N_2 fixation activity on the seed protein and oil contents

2.1.1 Estimation of N derived from nitrogen fixation (Ndfa), N derived from N fertilizer (Ndff) and N derived from soil (Ndfs) in seeds by monitoring $\delta^{15}N$

We used the most popular cultivar in Japan, Enrei, and its two near-isogenic lines (NIL) to evaluate the effect of nodule's N_2 fixation activity on seed protein and oil contents. One of the NILs [5, 6], En1282, was the no nodulating isoline [7], and another NIL, En-b0–1, was the hyper nodulating isoline [8]. As high soil nitrate levels inhibit the nodule formation and N_2 fixation activity of soybean plants [3], we applied different types of coated urea slow-release nitrogen fertilizers (CUSLNFs) having different lifespan to inhibit the N_2 fixation activity of plants for a certain period. The used CUSLNFs continuously emit N contained in them for 0 to 30 days for M5, 60 to 120 days for MS9, and 0 to 100 days for M15, respectively. We designated four types of experimental plots with different expected soil N levels (H, high or L, low) during the early and late periods of plant growth: the plots 'L-L', 'H-L', 'L-H', and 'H-H' were with no CUSLNF, M5, MS9, and M15, respectively. N_2 fixation of Enrei and En-b0–1 were expected to be suppressed during their working period. **Table 1** shows $\delta^{15}N$ values of matured seeds of the three NILs grown on the

Genotype	N Treatment			
	L-L	H-L	L-H	H-H
En1282	3.36	2.57	1.22	1.07
	(0.60)	(0.51)	(0.66)	(0.14)
Enrei	−0.20	2.12	0.33	1.88
	(0.36)	(1.36)	(0.14)	(0.36)
En-b0–1	−0.61	1.46	1.06	1.69
	(0.22)	(0.02)	(0.09)	(0.22)

The $\sigma^{15}N$ value of an urea coated slow-released nitrogen fertilizer (Meister 15) was −1.41.
$\sigma^{15}N$ values: Averages of duplicated samples. Parentheses indicate the SD (n = 2).

Table 1.
The $\sigma^{15}N$ values of mature seeds from plants of three genotypes of soybean.

four types of plots. We discriminated N derived from N_2 fixation, soil and CUSLNFs using the $\delta^{15}N$ values of seeds based on the authorized method [9]. Namely, ratios of seed N from the three origins in the NILs were estimated by the equations described in the footnotes of **Tables 2** and **3**. Since the En1282 plants utilized the mineral N that was available in the experimental fields, the $\delta^{15}N$ value of En1282 seeds of the L-L plot (3.36) was of the N supplied from the soil and the basal compound fertilizer. We distinguished the CULSNF-N from the N from the soil and compound fertilizer by using the $\delta^{15}N$ values of En1282 seeds of H-L, L-H, and H-H N plots then estimated the ratios of CULSNF-N as 16.6%, 44.8%, and 48.0%, respectively (**Table 2**). In the case of En-b0–1 plants grown on the L-L plot, the N from both the soil and the compound fertilizer was assumed to be negligible as %Ndfa of the plants was estimated to 118.1%. We presumed that the $\delta^{15}N$ value (−0.61) of En-b0–1seeds of L-L N plot was of the N from N_2 fixation. The contribution rates of the N_2 fixation in Enrei grown on the L-L, H-L, L-H, and H-H plots were 89.9%, 14.2%, 48.9%, and − 47.9%, respectively, and those of En-b0–1 were 100%, 35.0%, 8.9%, and − 36.5%, respectively (**Table 3**). The contribution rates of fixed-N ratio in the seeds from the plants of these nodulating genotypes grown on the H-H plot were negative values because these $\delta^{15}N$ values were higher than those of the En1282 plants. The contribution to the total N from the N_2 fixation in both Enrei and En-b0–1 was assumed to be zero. The number of nodules in a nodulating soybean

N	Ratio, %		Amount, gN/plant		
Treat.	Soil + BF	CUSLNF	Soil + BF	CUSLNF	Sum
L-L	100	0	0.63	0	0.63
H-L[§]	83.4	16.6	1.50	0.30	1.80
L-H[§]	55.2	44.8	1.20	0.98	2.18
H-H	52.0	48.0	1.32	1.22	2.53

The values were calculated by the equation described below under the assumption that the two types of CUSLNF, M-5 and MS-9, had the same $\sigma^{15}N$ values with that of Meister 15. The ratios of these N origins of seeds from plants of En1282 grown on the other N plots were estimated by using these $\sigma^{15}N$ values.
$\sigma^{15}N(En1282_{i-ii}) = (3.36) \times X_{i-ii}/100 + (-1.41) \times Y_{i-ii}/100$ ($X_{i-ii} + Y_{i-ii} = 100$), where X indicates the percentage of the sum of soil N and compound fertilizer N against the total N, Y represents the percentage of CUSLNF-N against the total N, and i–ii is the estimated N levels of the early and late periods of plant growth (H or L levels of N).

Table 2.
The ratios and amounts of N originated from the two origins, soil + compound fertilizer and CUSLNFs, in the total N in seeds from plants of non-nodulating genotype, En1282.

N	Genotype	Ratio, %	Amount		gN/plant			
Treat.		Soil + BF	SLNF	N_2 fix.	Soil + BF	SLNF	N_2 fix.	Sum
L-L	Enrei	10.1	0	89.9	0.28	0	2.48	2.75
	En-b0–1†	0	0	100	0	0	0.53	0.53
H-L§	Enrei	71.6	14.2	14.2	1.21	0.24	0.24	1.70
	En-b0–1	54.2	10.8	35.0	0.40	0.08	0.26	0.73
L-H§	Enrei	28.2	22.9	48.9	0.97	0.79	1.68	3.43
	En-b0–1	50.3	40.8	8.9	0.53	0.43	0.09	1.06
H-H	Enrei‡	76.9	71.0	−47.9	1.67	1.54	−1.04	2.17
		(52.0)	(48.0)	(0)	(1.13)	(1.04)	(0)	(2.17)
	En-b0–1‡	71.0	65.5	−36.5	0.95	0.87	−0.49	1.33
		(52.0)	(48.0)	(0)	(0.64)	(0.69)	(0)	(1.33)

†*En-b0–1 plants grown on L-L soil; it was assumed that N was only from N_2 fixation.*
‡*The numbers of each fraction of nitrogen origins in parentheses were calculated by the equation described in the text of Ref [5] in the cases in which N_2 fixation did not work.*
§*The values were calculated by the equation described in the text of Ref. [5] under the assumption that the two types of CUSLNF had the same $\sigma^{15}N$ values with that of Meister 15.*

Table 3.
The ratios and amounts of N originated from the three origins, soil + compound fertilizer, CUSLNFs, and N_2 fixation, in total N in seeds from plants of the two nodulating genotypes, Enrei and En-b0–1.

line T202 per plant during the seed maturation stages was affected by the type of applied CUSLNF (**Figure 1**) [10]. The same effects of CUSLNF was observed in Enrei (data not shown). Pot experiments under the conditions corresponded to the L-L and H-H plots showed that N_2 fixation activities of Enrei and En-b0–1 worked on L-L but did not work well on the H-H plot, respectively, judging from the changes in ureides' concentrations in xylem saps [6]. Thus, nodulation in Enrei was almost inhibited in the H-H plot supported the estimation that the amount of N from N_2 fixation was very low in this experiment. We also observed that the number

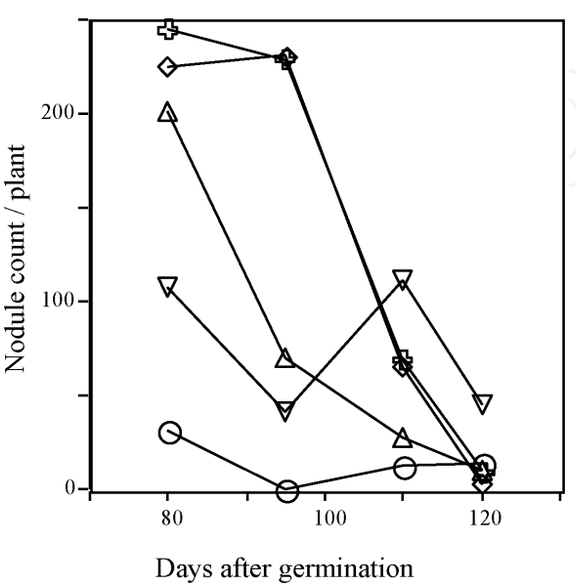

Figure 1.
Effects of N fertilizations on the changes in nodule counts per plant during seed maturation of cv T202. Symbols indicate soil type (fertilizers applied before planting) as follows circle.M-15; triangle, M-5; inverted triangle, MS-9; rhombus, urea; cross, no fertilizer.

of nodules in T202 grown in the H-L plot was rapidly decreased during the seed maturation period (**Figure 1**) [10]. The number of nodules of H-L was less than half of that of L-L at 95 days after germination, and we speculated that nodules in Enrei of the H-L plot did not develop well at the early stage of vegetative growth, resulted in much less N stored in nodules.

2.1.2 Coated urea slow-release N fertilizers differently affected seed chemical compositions and seed yields in the three NILs

Seed yields per plant of the 3 NILs are shown in **Figure 2**. Differences in seed yields of En1282 certified that fertilizers affected seed yield as expected and that the seed yields were proportional to the amount of N excreted from fertilizers. Seed yield of Enrei grown on the L-L plot was almost the same with that grown on the H-H plot, implying that the amount of N_2 fixation-derived N in seeds of the L-L plot was almost same with the amounts of fertilizer-derived N in seeds of the H-H plot. Seed yields of En-b0–1 were proportional to the amount of N applied from fertilizers, and this suggested N_2 fixation activity in En-b0–1 did not work well on this growth condition. This result is inconsistent with the low value of $\delta^{15}N$ value in seeds of the L-L plot and high ureides concentration in xylem saps from plants grown on the L-L condition in pot experiment.

The estimated amounts of N from the two origins (the soil with the compound fertilizer and the SLNF) in En1282, grown with the 4 N treatments, are listed in **Table 2**. Those from the three origins (the soil, the compound fertilizer, and N_2 fixation) in the two nodulating genotypes grown with the 4 of N conditions are listed in **Table 3**. In En1282, the amounts of N/plant from both the soil and compound fertilizer were similar among H-L, L-H, and H-H, and the estimated

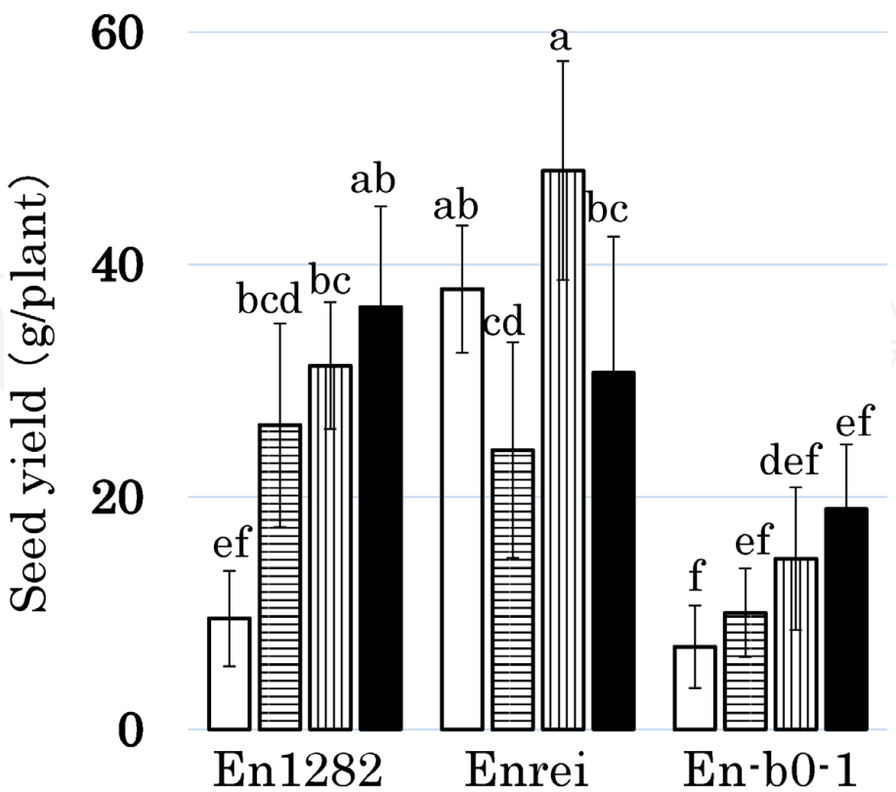

Figure 2.
Effects of the N fertilizations on the seed yields of three genotypes of soybean. Symbols indicate the types of N treatment where plants were grown: white, L-L; horizontal stripe, H-L; vertical stripe, L-H; black, H-H. Different letters indicate significant differences between genotypes and N treatments.

amounts of N/plant from the CUSLNF were 0.30, 0.98, and 1.22 g for the plants grown on H-L, L-H, and H-H, respectively. In Enrei, the amount of N/plant from N_2 fixation of L-H was 1.68 g, which was two-thirds of that on L-L. In contrast, the amount of N/plant from N_2 fixation in En-b0–1of L-H was 0.09 g/plant, which was one-fifth of the amount from the plants grown on L-L.

In the Enrei and En-b0–1 plants grown on H-H, the amount of N from N_2 fixation was negligible, and the amount of N from the CUSLNF was the highest among the N amounts from the 4 of N conditions.

For Enrei, the N supply from the soil had no apparent effect on the seed yield or seed weight (SW). It means that N derived from N_2 fixation compensates for the low supply of soil N for plant growth. Among the Enrei plants grown on the 4 types of N conditions, we observed a lower yield, a smaller SW, and a smaller number of seeds on H-L (**Figure 2**). Our observation that the $\delta^{15}N$ values of mature seeds of H-L was the highest among the Enrei plants grown on the 4 types of N conditions (**Table 1**), which implies that the ratio of N from N_2 fixation to the total N of the plants grown on H-L was less than that of the Enrei plants grown in the other conditions. Enrei plants develop nodules during the early stage of plant growth, and soil nitrate inhibited nodule growth of soybean plants [11]. The $\delta^{15}N$ value of seeds from plants grown on L-H (where changes in the amount of N released from the CUSLNF was opposite to the changes in the plants grown on H-L) was slightly higher than that of the plants grown on L-L. Considering this observation and the result that En1282 assimilated N well in the late growth stage (**Table 2**), the importance of N assimilation at the late plant growth was suggested. Moreira *et al* reported that foliar N application at the pod formation period increased seed yield under a certain environmental condition [12]. The work of Takahashi *et al* (1991) showed that the effectiveness of N fertilization at the late period of plant growth of soybean by the deep placement of N fertilizers that improved seed yields [13].

The inter-relationships among the seed protein content, seed oil content, and SW are illustrated for the three genotypes described in 2.1.1. (**Figures 3** and **4**). The protein content was proportional to the SW in En1282 and Enrei (**Figure 3A**). There was no such a relationship in En-b0–1. The protein content inversely correlated with the seed oil content in En-b0–1 (**Figure 3C**). The seed oil content uncorrelated with the SW and the protein content in En1282 and Enrei (**Figure 3B,C**). En-b0–1 seeds on L-L showed the highest protein content and the lowest oil content among the three genotypes (**Figure 3C**). The results of En1282 of L-L was opposite to those (**Figure 3C**).

The effects of the N treatments on the interrelationship between protein and oil contents in seeds differed among the three genotypes (**Figure 4**). The seed protein contents and seed oil contents of En1282 of L-H and H-H were higher and lower than those of L-L and H-L, respectively. The reverse was true for En-b0–1 on the same types of N conditions. The seed oil contents in Enrei of the 4 of N conditions were almost the same. The seed protein content of Enrei of L-L was the highest among the 4 N conditions, and the protein and oil contents in seeds of the three genotypes grown on H-H were almost the same.

We calculated the amounts of seed protein and seed oil by multiplying the contents of protein or oil by the SW (**Figure 5**). In En1282, the amount of oil per seed was proportional to the amount of protein per seed irrespective of the N treatment types (the *r* of the coefficient line was 0.998). The relationship between the amounts of oil and protein per seed seemed to be dependent on the N treatments in the two nodulating genotypes. The trends of the oil and protein amounts in Enrei and En-b0–1 seeds of L-H and H-H were similar to those of H-L and L-L. In other word, higher N_2 fixation activity of Enrei plants decreased amount of oil accumulation in seeds.

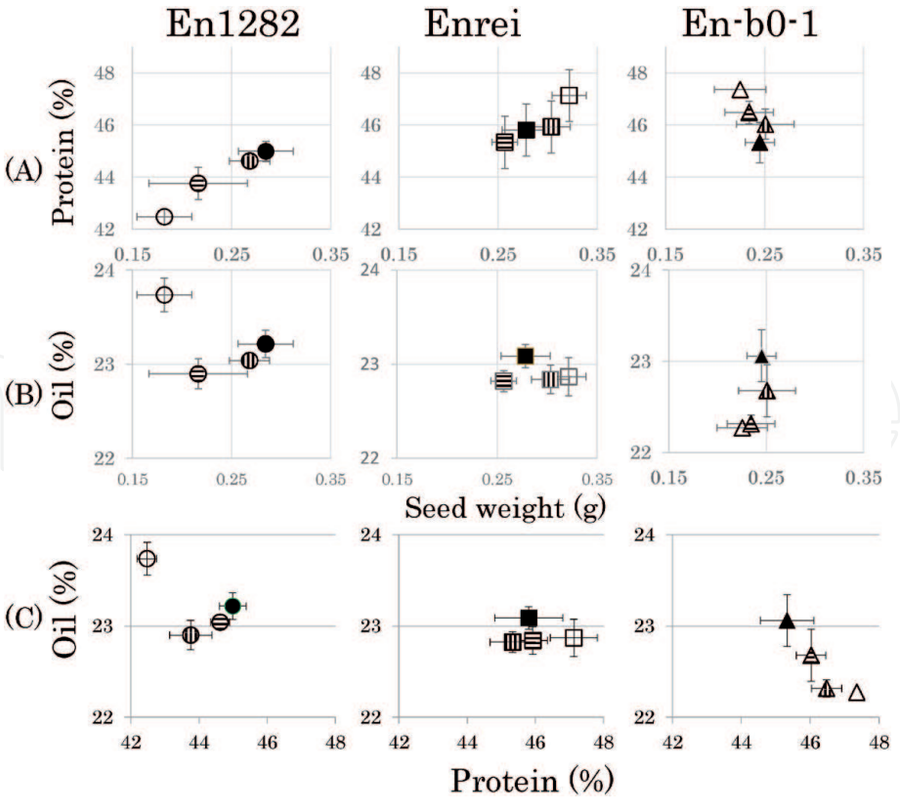

Figure 3.
Effects of N fertilizations on the contents of protein and oil in seeds from plants of three genotypes of soybean. (A) Relationships between protein content and seed weight. (B) Relationships between oil content and seed weight. (C) Relationships between oil and protein content. Shapes of symbols, circles, squares and triangles, indicate the CVs, En1282, Enrei and En-b0–1, respectively. Patterns inside symbols indicate types of soil where plants were grown as follows: white, L-L; vertical stripe, H-L; horizontal stripe, L-H; black, H-H. Correlation coefficients between protein content and seed weight from plants of En1282 and Enrei were 0.982 and 0.907, respectively. Significance levels were 0.02 and 0.10 for En1282 and Enrei, respectively. Correlation coefficient between protein content and oil content from plants of En-b0–1 was 0.924, and it's significance level was 0.10.

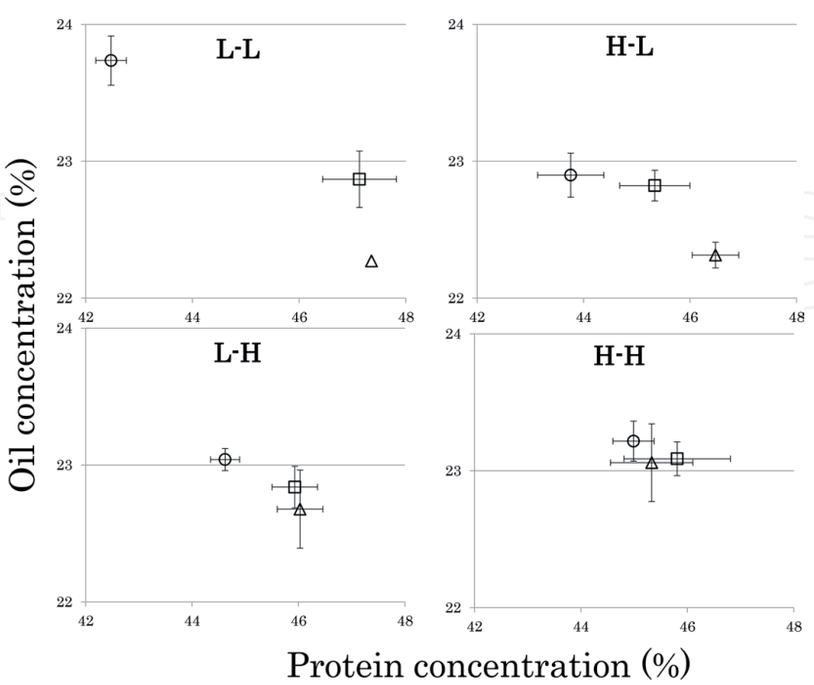

Figure 4.
Interrelationships between the protein content and oil content in seeds from plants of the three soybean genotypes. The four panels indicate the N plots as follows. Left-upper panel: L-L, right-upper panel: H-L, left-lower panel: L-H, right-lower panel: H-H. shapes of symbols indicate the same genotype as those described in the ***Fig.*** *2 legend.*

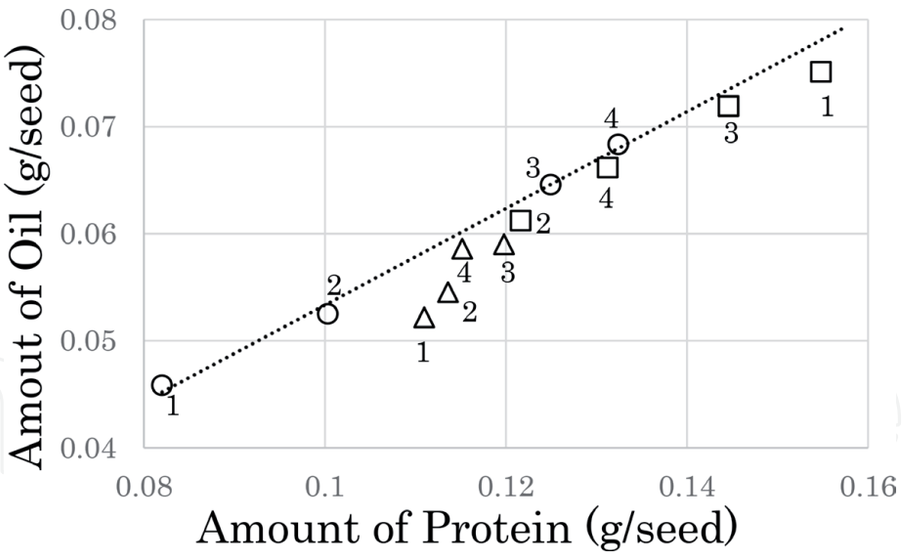

Figure 5.
Effects of the N fertilizations on the amounts of protein and oil per seed from plants of the three soybean genotypes. Shapes of symbols indicate the same genotype as those described in the ***Fig. 1*** *legend. Numbers beside marks indicate types of N plots where plants were grown as follows; 1:L-L, 2:H-L, 3:L-H, and 4: H-H. the* dotted line *indicates the coefficient line between the amount of oil per seed and the amount of protein per seed from En1282 plants (p = 0.01).*

2.2 Effect of different types of N fertilizers on seed chemical composition suggested independently regulated accumulation of protein and oil in soybean seeds

T202 plants were grown on 4 types of N fertilization conditions, where soil N levels were changed in different manners [14]. The 4 N fertilization types were (1) no N fertilizer, (2) urea, (3) M-5, and (4) M-15. The N fertilization condition (1), (3), and (4) were similar to L-L, H-L, and H-H described in 2.1, respectively. Plants of T201, a non-nodulating NIL of T202, were also grown on the 2 types of N fertilization conditions (3) and (4). Firstly, we investigated on the relationship between seed protein content and seed oil content per individual plant basis (**Figure 6**). T202 and T201 exhibited very similar protein and oil contents under the N condition (4), being due to less N_2 fixation in T202. T202 under the N condition (2) exhibited similar protein contents and slightly less oil contents with those of T202 and T201 of (4). This would be due to the different characteristics of M-15 and urea as N fertilizers. T202 and T201 of (1) and (3), both of which offer low nitrogenous conditions at the seed maturation stages, exhibited negative correlations between protein contents and oil contents in each condition, implying these correlations are dependent on the availability of N from soil.

Next, we investigated on the relationship between amounts of protein and oil per seed under the 4 nitrogenous conditions (**Figure 7**). Notably, observed relationships between amounts of protein and oil were exactly opposite to those of seed protein and seed oil contents of individual plants. Namely, each plant samples of (2) and (4) showed positive correlation relationships between amounts of protein and oil (**Figure 7**). In addition, a weaker positive correlation between amounts of protein and oil per seed was observed in T202 grown on the N condition (3). T201 of the N condition (4) exhibited a positive correlation between amounts of protein and oil per seed; it was similar to that of T202 grown on the same N condition. T202 of the N condition (1) did not show any correlation between amounts of protein and oil per seed, being contrast to that of protein and oil contents among individual plants. This could be due to the low variation of the amounts of protein and oil per seed in these samples. Hence, these results suggested that the observed negative

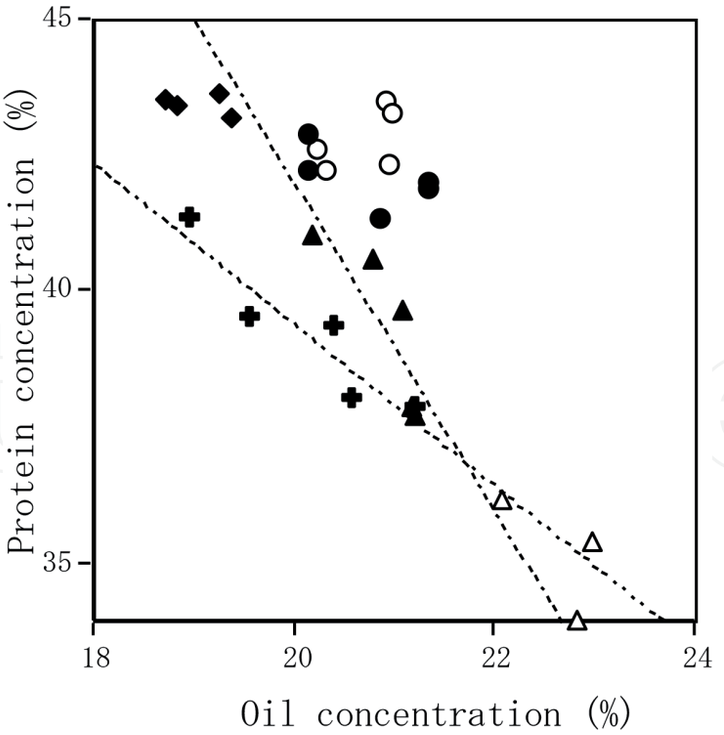

Figure 6.
Effects of soil N level and nodulation on concentrations of protein and oil in soybean seeds. Symbols indicate soil type as follows: circle, M-15; triangle, M-5; rhombus, urea; cross, no fertilizer. Solid and open symbols indicate nodulated cv, T202 and non-nodulated cv T201, respectively. Equations of correlation lines were as follows: line a, Y = -3.01X + 102.19 (r^2 = 0.709) for seeds from T202 on M-5 soil and line b, Y = -1.42X + 68.93 (r^2 = 0.864) for those from L soil, respectively. Y and X denote protein and oil concentrations, respectively.

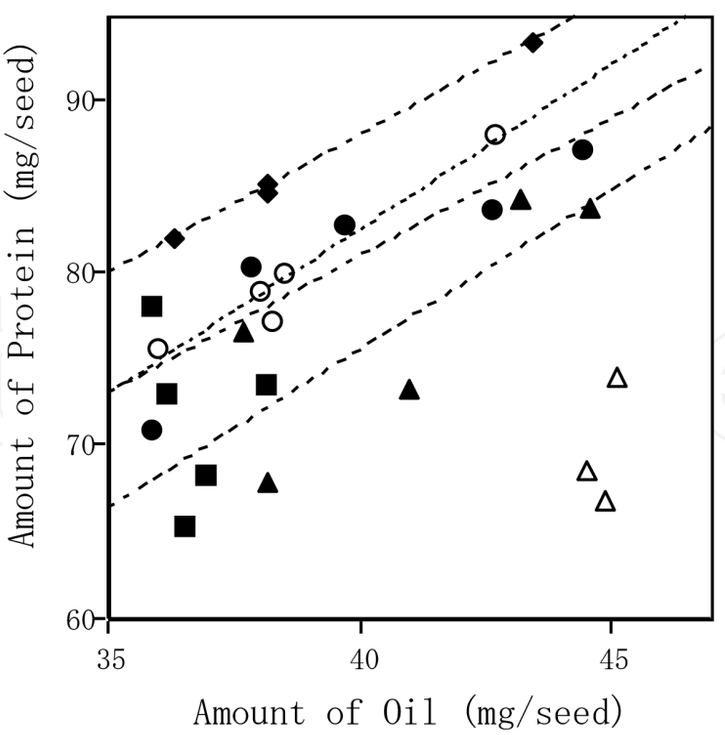

Figure 7.
Effects of soil N level and nodulation on amounts of protein and oil per seed in soybean seeds. Symbols indicate soil type as follows: Circle, M-15; triangle, M-5; rhombus, urea; cross, no fertilizer. Equations and coefficients of correlation lines were as follows: Line a, Y = 1.59X + 17.34 (r^2 = 0.817) for seeds from cv T202 on M-15 soil; line b, Y = 1.84X + 1.1.78 (r^2 = 0.632) for those on M-5 soil; line c, Y = 1.62X + 23.39 (r^2 = 0.997) for those on urea-soil, and line d, Y = 1.91X +6.21 (r^2 = 0.946) for those of cv T201 on M-15 soil, respectively. Y and X denote seed contents of protein and oil, respectively.

correlations of protein and oil contents in the N condition (1) was caused by different seed protein amounts per seed, which were made by receiving insufficient N to immature seeds. The observation that T201 of the N condition (3) (where offers low N supply during seed maturation) exhibited different protein contents and similar oil contents among individual plants implied that insufficient N supply did not affect oil accumulation in seeds.

The regression lines between amounts of protein and oil per seed in T202 of (2), (3), and (4) had similar slopes. This result implied that carbon transported into immature seeds was distributed to the biosynthesis of protein and oil in the same ratio among these plants. In addition, the Y-intercepts of the regression line of T202 under the N conditions (2) and (3) were larger and smaller than those of (4), respectively. Since the amount of N supplied to immature seeds from vegetative tissues would be increased in the order of (1), (3), (4), and (2), the differed Y-intercepts implicate another mechanism which is independent from carbon partitioning for the biosynthesis of amino acids and fatty acids. As mentioned in 2.3, seed oil accumulation ceased before seed maturation, but seed protein accumulation continued till seed maturation. The difference in the Y-intercept may be due to the different amount of accumulated proteins after the oil accumulation has ceased.

2.3 Inhibited N_2 fixation by N application at the flowering stage did not promote the protein accumulation but did the oil accumulation and dehydration in soybean seeds

2.3.1 Ammonium sulfate and NaCl applications at the flowering stage differently affects the protein and oil contents in soybean seeds

Soybean plants were grown on soil, which allows developing nodules well, and high amount of ammonium sulfate (AS) was applied (10 g of solid AS was spread around a plant) to T202 at the flowering stage [14]. We observed that the AS application did not change the protein content per seed but increased the oil content although seed protein content in AS-applied T202 individuals was lower than that in control (**Table 4**) [11]. The ureide concentrations in xylem saps from the NaCl and AS applied plants were lower than untreated ones in the same extent with each other till 25 days after the applications, which implied that plants of both treatments were suppressed their N_2 fixation activities to the same level as each other (**Figure 8**). Oil contents per seed of both treatments were more than those of the no treatment (**Table 4**). On the other hand, protein contents per seed from the AS treated plants were comparable to those from the no treated plants, which was quite different from those from the NaCl treated plants, of which protein content was

	Concentration, mg/g DM		Content, mg/seed		Seed weight
	Protein	Oil	Protein	Oil	g/seed
No	411	230	69.0	38.6	0.168 (0.022)
AS	391	239	69.6	42.5	0.178 (0.020)
NaCl	388	239	65.6	40.4	0.169 (0.020)

In the seed weight column, the standard deviations of seed weight from 70 grains are indicated in parentheses. Mature seeds were harvested 72 days after application. Protein and oil contents per seed were calculated by multiplying their concentrations by seed weight, respectively.

Table 4.
Effects of application of ammonium sulfate (AS) and NaCl at flowering stage on seed storage composition of soybean plants (cv T202).

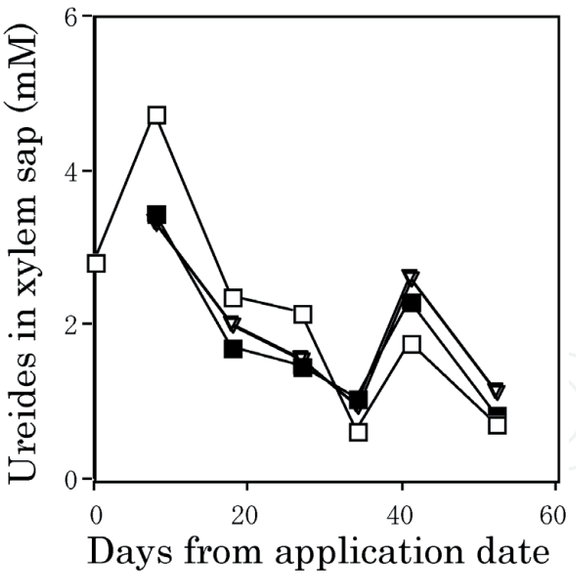

Figure 8.
Effects of application of ammonium sulfate and NaCl at the flowering stage on concentration of ureides in xylem saps from nodulated soybean plants (cv T202).Symbols indicate chemicals applied as follows: Open square, no treatment; solid square, ammonium sulfate; and rectangular triangle, NaCl, respectively. Values of ureides in the xylem saps were given as means of those from two plants from each treatment groups.

lower than those from the no treated plants (**Table 4**). These results suggested that applied AS compensated the decrease in the amount of N from N_2 fixation. That suppression of N_2 fixation activity caused an increase in the amount of oil in seeds.

2.3.2 Different effects of ammonium sulfate application at the flowering stage on the accumulation of protein and oil in soybean seeds

Application of AS at flowering stage to Enrei and Tamahomare decreased protein and increased oil contents in matured seeds of both CVs [15]. Averaged protein and oil contents in seeds from the AS dressed plants were 2% lower and higher than those from the undressed plants, respectively, in Enrei. Accumulation profiles of protein and oil per seed during their ripening period were quite different from each other (**Figures 9** and **10**). The regression curve of the increase in the amount of protein per seed during seed maturation was almost identical between plants that received N at the flowering stage and those that did not. Contrary to this, the regression curves of oil content per seed showed seeds from N applied plants accumulate oil faster than those of control plants did. Seeds of both N treatments stopped in increasing oil content at the late period of seed maturation. N applied plants showed a faster seed dehydration rate than the control plants on the results of changes in the water contents of seeds during seed maturation (**Figure 11**). Matured seeds from N applied plants had less protein content than those from N unapplied ones. An increase in seed weight was higher in N applied plants than those in the control plants. These results implied that a high amount of N application at the flowering stage suppressed N_2 fixation activities of nodules, causing a decrease in sucrose consumption by nodules. The dehydration rate of seeds from N applied plants was faster than those from N unapplied plants. The fact implied that the accumulation rate of storage compounds, protein and oil, in seeds from N applied plants was faster than those from N unapplied plants. In other words, a higher amount of C (sucrose) was supposed to supply to maturing seeds in the case of N applied plants than in the case of N unapplied ones. Sudden suppression of N_2 fixation of nodules by ammonium sulfate application to plants was supposed to cause the decreased consumption of sucrose in nodules and the increase in the amount of sucrose imported into maturing seeds. Thus seeds increased the oil content resultantly.

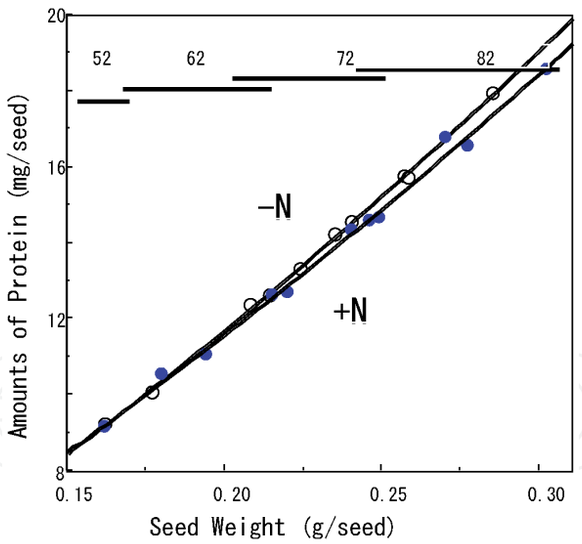

Figure 9.
Effects of N application at the flowering stage on the increase of protein content in soybean seeds during maturation. Open and solid symbols show the protein content per seed from the N undressed and dressed plants, respectively. Symbols indicated the days from the dressed day as follows: Circle, 52; square, 62; triangle, 72; inverted triangle, 82, respectively. Regression curves for values of the each treatment are shown. Equations for the curves are $Y = 56.90X^2 + 45.40X + 0.35$ ($r^2 = 0.999$) and $Y = 47.96X^2 + 44.90X + 0.69$ ($r^2 = 0.995$) for the protein contents of undressed and dressed plants, respectively. Y and X means amount of proteinous N (mg) in a seed and seed dry weight (g), respectively.

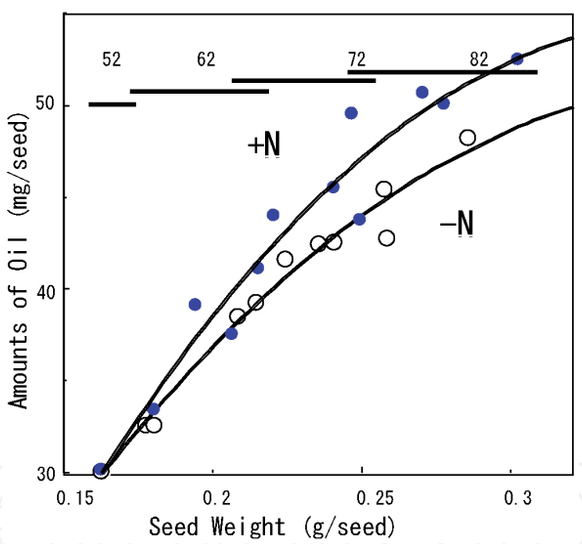

Figure 10.
*Effects of N application at the flowering stage on the increase of oil content in soybean seeds during maturation. Symbols were the same as those in **Figure 8**. Regression curves for values of the each treatment are shown. Equations for the curves are $Y = -0.495X^2 + 0.366X - 0.0163$ ($r^2 = 0.977$) and $Y = -0.657X^2 + .467X - 0.0284$ ($r^2 = 0.941$) for the oil contents of undressed and dressed plants, respectively. Y and X means amount of oil (mg) in a seed and seed dry weight (g), respectively.*

2.4 Different effects of CUSLNFs on seed protein concentrations of plants producing high and low protein content seeds – Accumulated proteins in nodules may be a factor to affect seed protein content

Plants of 13 CVs producing low-, medium- and high- protein content seeds were grown on similar conditions to the L-L, H-L, and H-H plots described in 2.1.1, and the protein and oil contents of harvested seeds were compared (**Figure 12**) [10].

Values subtracted seed protein contents from plants grown on H-H soil from those grown on L-L soil were compared among 13 CVs based on the protein concentrations

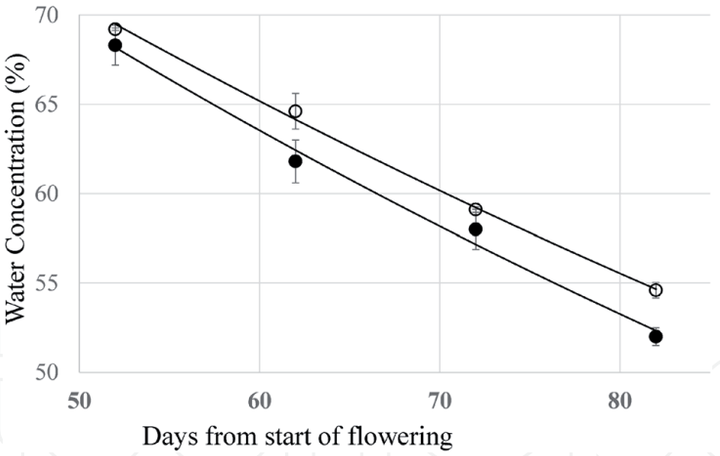

Figure 11.
Effects of N application at the flowering stage on the changes in water content of soybean seeds during maturation. Open and solid symbols show the water contents from the N undressed and dressed plants, respectively.

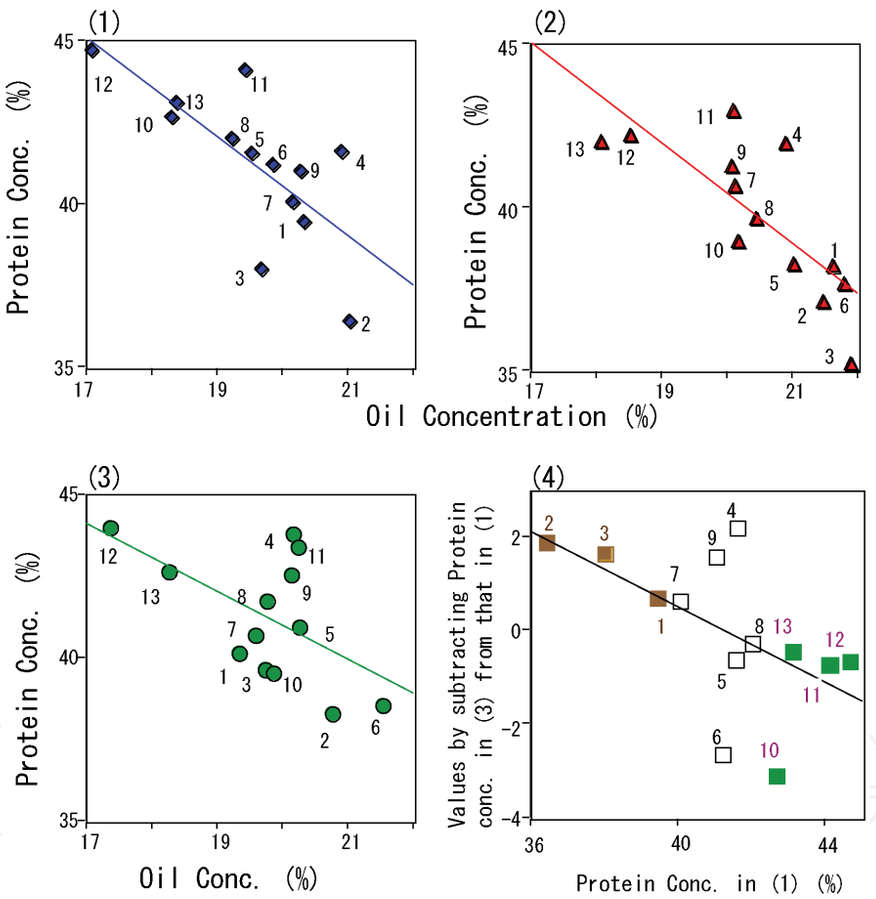

Figure 12.
Different effects of CUSLNFs on the contents of protein and oil in mature seeds of 13 cultivars of soybean. Left-upper panel, (1) interrelationships between the protein content and oil content in seeds from plants grown on L-L soil. Right-upper panel, (2) interrelationships between the protein content and oil content in seeds from plants grown on H-L soil. Left-lower panel, (3) interrelationships between the protein content and oil content in seeds from plants. Right-lower panel, (4) interrelationship between the value of protein content of seeds from plants grown on H-H soil (3) subtracted from that on L-L (1) soil and protein content of seeds from plants grown on L-L soil (1). Numbers in the figure indicate the cultiver names as follows: 1, Akishirome; 2 Ginrei; 3, Tamahomare; 4, Ohtsuru; 5, Kyu-kei 273; 6, Tamamasari; 7, Nishimusume; 8, Asagoao; 9, Mizukuguriokute; 10. Toyoshirome; 11, Fukuyutaka; 12, Miyagiaosho; and 13, Bunjyocha. Cvs of which number 1 to 3, 4 to 9 and 10 to 13 were classified to those producing low (less than 40%), medium (40 to 42%) and high (more than 42%) protein content seeds, respectively, based on the results shown in panel (1). Colors of symbols in panel (4) indicate the class of seed protein content of CV as follows: brown, low; white, medium; green, high. The X-axis and Y-axis coefficient lines for each panel and their coefficients of determination are as follows: (1) $Y = -1.519X + 70.886$ ($r^2 = 0.524$), (2) Y—$1.5144X + 71.301$ ($r^2 = 0.583$), (3) $Y = -1.038X + 61.712$ ($r^2 = 0.312$), and (4) $Y = -0.403X + 16.604$ ($r^2 = 0.320$).

of seeds from plants grown on L-L soil (**Figure 12D**). A negative correlation between the subtract values and seed protein contents of L-L soil was observed. Seed protein contents in 3 low-protein CVs grown on L-L soil were higher than those of H-H soil. The reverse was true for four high-protein CVs. Differences in seed protein contents between plants producing low and high protein seeds were possibly ascribed to the amount of N that immature seeds received from nodules and soil.

Protein contents correlated with oil ones in all cases, and coefficients of determination were 0.724, 0.770, and 0.559 for seeds grown on the L-L, H-L, and H-H conditions, respectively. The coefficient between protein and oil concentrations from plants grown on H-H soil was lowest, and that of those grown on H-L soil was highest among plants grown on the three types of soils. This result suggests a higher amount of N supply from nodules or soil during seed maturation promotes protein accumulation but does not oil accumulation because plants grown on the H-L soil might have fewer nodules than those on L-L, and they absorb less soil N than those on the H-H soil during reproduction stage.

Different seed weight distributions between a low protein cultivar Tamahomare (TH) and those of a high one Fukuyutaka (FU) supported this idea. Plants of TH grown on L-L had smaller seeds than those on H-H. The reverse was true for FU (**Figure 13**). Seeds of CV FU grown on H-L had similar seed weight distribution to that on L-L. On the other hand, seeds of CV TH grown on H-L were smaller than those on L-L, judging from their seed weight distribution profiles. These results suggested that immature seeds did not receive enough amount of N from vegetative tissues including nodules for the demand of seeds in the case of CVs producing low protein content seeds grown on L-L. Such plants grown on H-H absorbed and metabolized fertilizer-derived N from soil enough for immature seeds' demand. These results suggested that plants of CVs producing high protein content seeds grown on L-L had enough amount of N in the vegetative tissues for the need of immature seeds. These results also implied that those grown on H-H where nodulation and N_2 fixation activity of plants were suppressed by soil N did not have enough amount of N in the vegetative tissues for the demand of immature seeds.

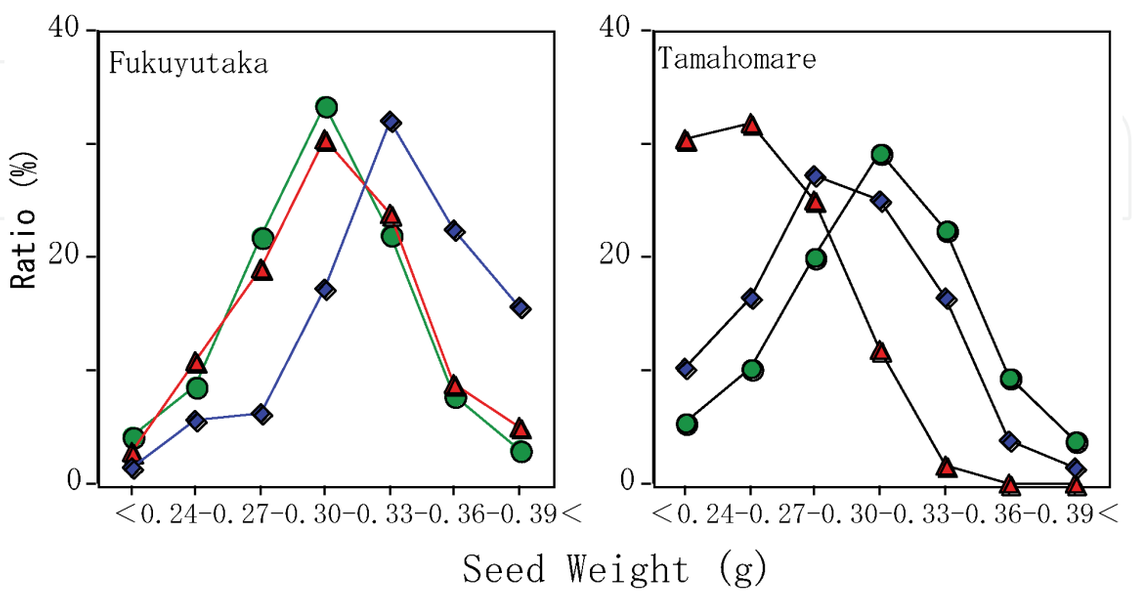

Figure 13.
Differences in the seed weight distributions from plants grown on three types of UCSLFs between two cvs producing seeds of high and low protein contents. Left and right panels show ratios of seed counts per seed weight ranges as a cv Fukuyutaka producing seeds of high protein content and a cv Tamahomare producing those of low protein content, respectively. Symbols indicate the soil types as follows; rhombus, L-L; triangle, H-L; circle, H-H.

3. Carbon metabolism in maturing seeds is affected by the supply of N from source organs-role of phospho*enol*pyruvate carboxylase (PEPC) in the accumulation of protein

3.1 Background for the research on the role of PEPC in the synthesis of storage protein in soybean seeds

Seed storage compounds in soybean, proteins and oils, are synthesized from substances transported from vegetative tissues, leaves, roots, and nodules. The primary forms of the substances are sucrose, ureides, and Asn [3]. Amino acids constituted storage protein are synthesized by introducing amino acid residues into organic acids in cotyledon during seed maturation. This fact implied two factors: amounts of organic acids and amino residues formed from the imported substances affect the protein content of soybean seeds. Since organic acids are utilized as the substrate for the synthesis of both fatty and amino acids, the inverse correlation between contents of protein and oil in soybean seeds [1] could be caused by the competition of fatty and amino acid synthesis. Non-photosynthetic type Phospho*enol*pyruvate carboxylase (EC 4.1.1.31, PEPC) was thought to play the anaplerotic role in the supply of organic acids [16]. Immature crop seeds have a high activity of the enzyme, and its role in maturing seeds was assumed to refix CO_2 formed from respiration in seeds [17].

Multiple types of PEPC isogenes were encoded in plant genomes. These enzyme genes were categorized into plant-type and bacterial-type isogenes [18]. Plant-type isogenes were further subdivided into C_4 and other (C_3) type. Subsequent sections present our research on soybean seed PEPC over time and discuss the role of PEPC isozymes in the synthesis of storage proteins.

3.2 Relationships of PEPC activity and contents of storage compounds, protein and oil, in mature soybean seeds

High CO_2 fixing activity was observed in immature cotyledons of soybean seeds under an unilluminated condition in a study [K. Tanaka, personal communication], which evaluates the photosynthetic activity of immature soybean seeds [19]. In cotyledon, ribulose 1,5-bisphosphate carboxylase (RuBPCase) gradually decreased its activity during seed maturation, whereas PEPC kept its activity high during seed maturation. PEPC enzyme rapidly decreased its activity between 3 and 9 days after germination [20]. These results implied the engagement of PEPC in the accumulation of storage compounds. As the enzyme activity was kept in matured soybean seeds, the enzyme activity and contents of storage compounds, i.e., protein and oil, were compared among seeds from plants of 13 types of seeds in 11 CVs grown in 12 prefectures of Japan (**Figure 14**) [21]. The enzyme activity was positively and negatively proportional to the protein and oil contents of seeds, respectively. This observation suggested PEPC in immature soybean seeds plays a role in the supply of carbon skeleton to synthesze of amino acids.

PEPC activity in maturing rice seeds increased its activity by the addition of N fertilizer [22]. In soybean, N fertilizer application was thought to be insufficient to the enzyme activity in seeds because soybean plants had nodules and supply N compounds in nodules to maturing seeds [3]. When N contents in leaves and PEPC activity in maturing seeds simultaneously were compared, they were proportional with each other (**Figure 15**) [10]. Leaves gradually lose their greenness by exporting amino acids to developing seeds during seed maturation, which means leaves lose photosynthetic activity. This observation suggested that PEPC in soybean seeds changed its activity responding to N supply from vegetative tissues and that PEPC plays an essential role in the amino acid synthesis for storage protein. One of PEPC

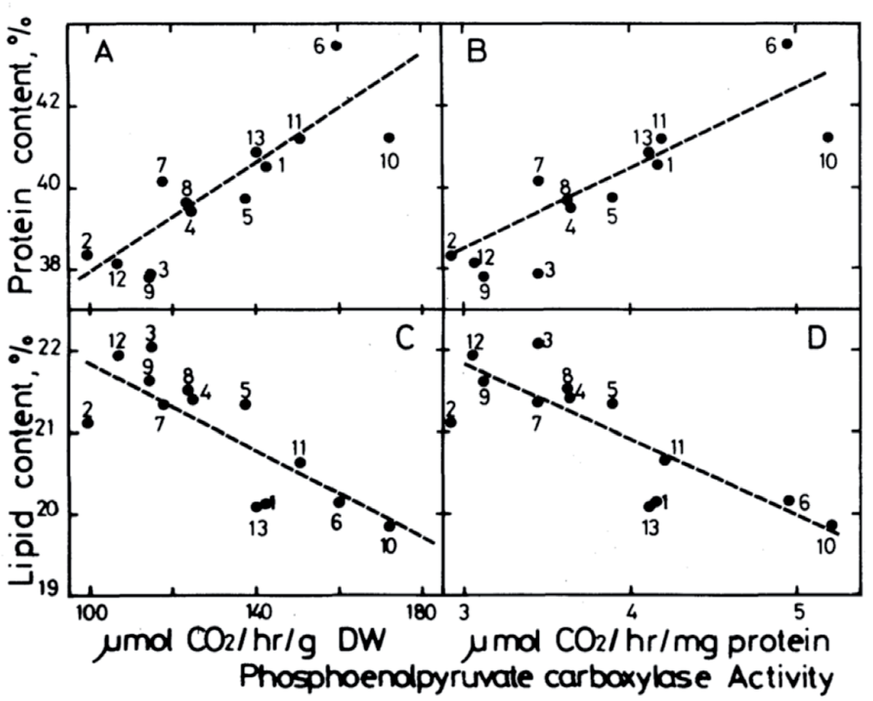

Figure 14.
Relationships between PEPC activity and contents of protein and lipid in soybean seed. Left-upper panel A: Relationship between PEPC activity per 1 g of dry seed (X) and protein content (Y) in soybean seed. Right-upper panel B: Relationship between PEPC activity per 1 mg of soluble protein (X) and protein content (Y) in soybean seed. Left-lower panel C: Relationship between PEPC activity per 1 g of dry seed (X) and lipid content (Y) in soybean seed. Right-lower panel D: Relationship between PEPC activity per 1 mg of soluble protein (X) and lipid content (Y) in soybean seed. Cultivar names and produced prefectures of soybean seeds harvested in 1986 are as follows: 1, Fukuyutaka (Fukuoka); 2, Tamahomare (Yamaguchi); 3, Akishirome (Yamaguchi); 4, Akishirome (Hiroshima); 5, Akiyoshi (Kagawa); 6, Enrei (Nagano); 7, Enrei (Niigata), 8, Tachisuzunari (Tochigi); 9, Suzuyutaka (Yamagata); 10, Miyagishirome (Miyagi); 11, Shirosennari (Akita); 12, Nanbushirome (Iwate); 13, Okushirome (Aomori). Equations and their correlation coefficients of the X-axis and Y-axis coefficient lines for A, B, C and D panels, respectively are as follows: Y = 0.062X + 31.727 (r = 0.8395); Y = 1.99X + 32.29 (r = 0.8660); Y = −0.029X + 24.884 (r = −0.8411); and Y = −0.91X + 24.35 (r = −0.8494).

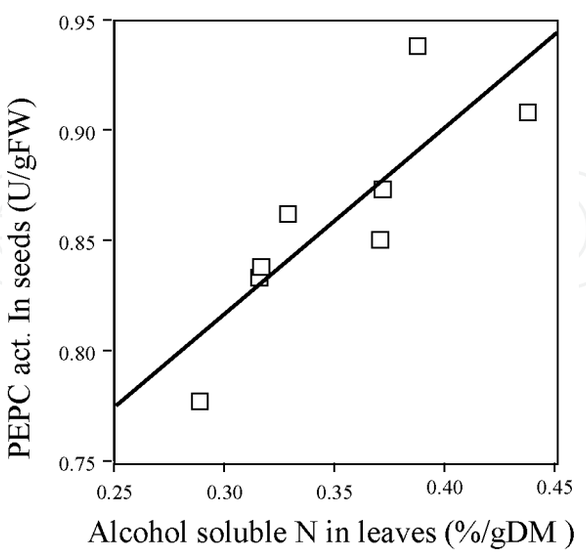

Figure 15.
Relationship between alcohol soluble nitrogen (amino acids) content in leaves and PEPC activity in immature seeds. The coefficient line and its coefficient of determination are as follows: Y = 0.846X + 0.564 (r^2 = 0.703).

isogenes was expressed in immature seeds and other vegetative tissues of soybean plants [23]. Another PEPC isogene expressed in soybean maturing seeds was identified together with the isogene mentioned above [24]. In recent, it appeared that ten

PEPC isogenes were encoded in the soybean genome [25]. It might be likely that multiple PEPC supports soybean seed metabolism during seed development.

3.3 Roles of several PEPC isoforms in maturing soybean seeds starch is a significant carbon source of carbon in protein biosynthesis during the late maturation period in soybean seeds

3.3.1 Comparison of PEPC activity, contents of protein and oil in seeds in high- and low-protein CVs using principal component analysis

We applied principal component analysis (PCA) to evaluate the interrelationships among the four factors of maturing seeds, contents of protein and oil, PEPC activity, and seed weight (**Figure 16**) [26]. The enzyme activity was significantly

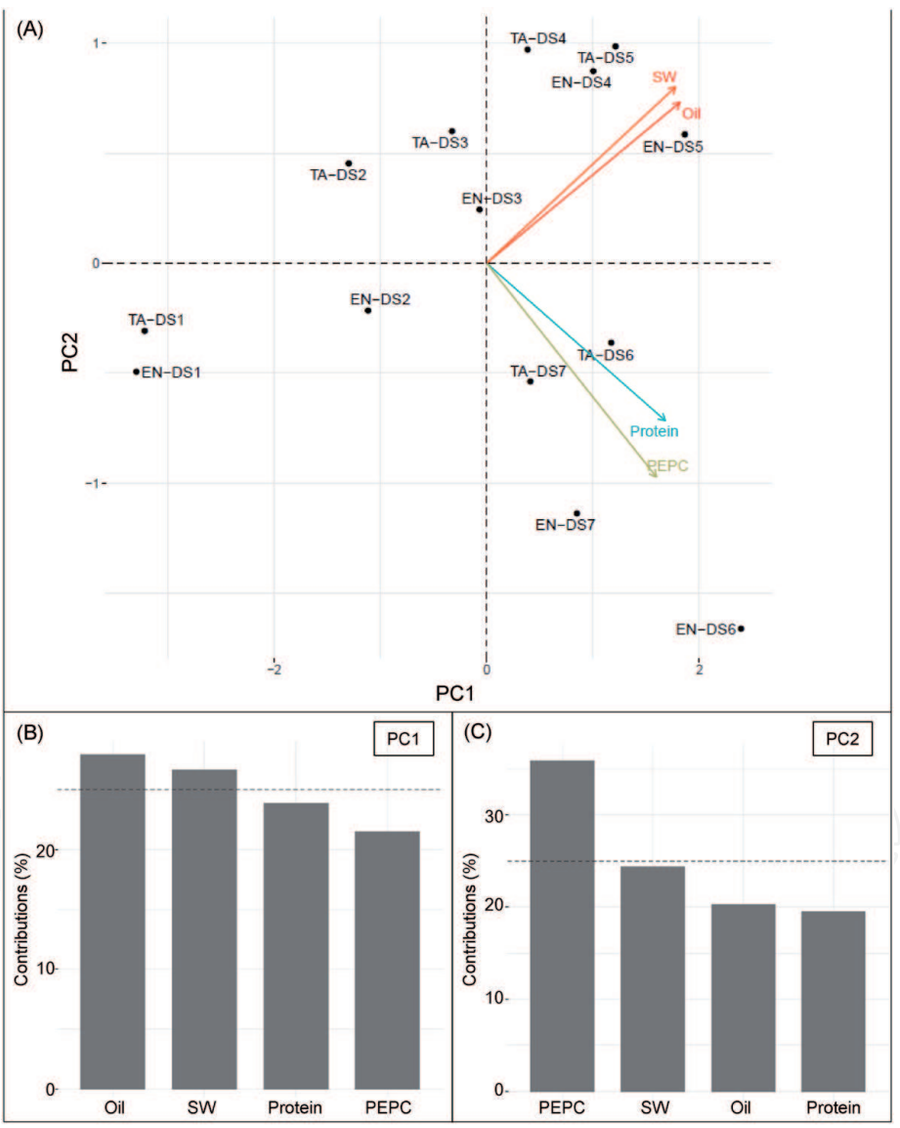

Figure 16.
The results of PCA in seed compositions, protein, oil, seed weight and PEPC activities during seed maturation. Characters and numerals show the name of cultivars and stage of samples, respectively. The characters EN and TA indicate Enrei and Tamahomare, respectively. The numerals with DS indicate the stage of samples. A: A biplot of the two major principal components, representing a distribution of the samples analyzed. Marks in black indicate samples, and arrows indicate the directions of protein content (protein), oil content (oil), seed fresh weight (SW), and PEPC activity per seed (PEPC). Horizontal- and vertical- axes represent principal component 1 (PC1) and principal component 2 (PC2), respectively. PC1 and PC2 explain 73.6 and 16.4% of the data variances, respectively. B and C: Contributions of the variables on PC1 and PC2, respectively. Dot lines indicate the averaged values.

associated with protein content but not with oil content. The oil content was associated with seed weight. Immunological assay on PEPC protein contents using antibodies for some PEPC isozymes showed plant-type ones expressed during all stages of seed maturation, and Gmppc2 expressed at the late maturation stage (**Figure 17**). The most critical period in the seed maturation period on the relation between protein content and PEPC activity was the late stage of seed maturation, DS6, in our discriminating seed growth stages.

Characteristic physiological changes of soybean plants are the loss of leaves and decrease in starch contents of seeds [27]. We observed that oil accumulation ceased at the late seed maturation period, and protein accumulation continued till seed maturation. Together, we proposed that PEPC plays a role in the supply of carbon skeleton of amino acids (organic acids) formed by the degradation of starch in seeds.

3.3.2 PEPC isogenes exhibits divergent expression patterns during seed maturation- Gmppc2 isogene is possibly a useful marker for improving seed protein content

The ten soybean PEPC isogenes showed different gene expression characteristics in developing seeds from each other [28]. Notably, one PEPC isogene *Gmppc2*

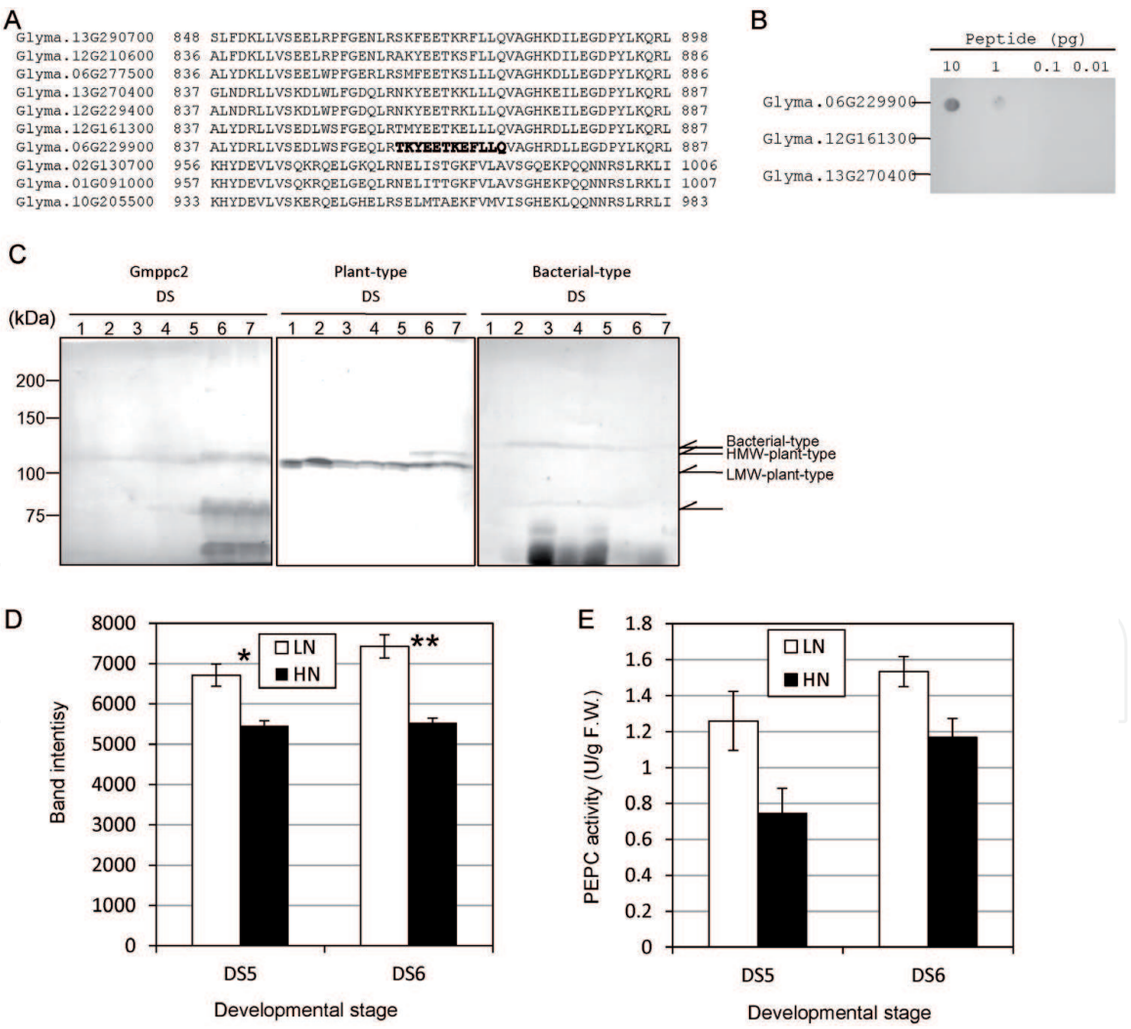

Figure 17.
*The expression of PEPC in immature soybean seeds. A: Partial sequence alignment of the ten PEPC isoforms of the C-termini. Bold letters: The region that was used to raise a Gmppc2-specific polyclonal antibody. B: Dot blot assay to determine the specificity of the Gmppc2 antibody. C: The protein expression patterns of Gmppc2, plant-type PEPC, and bacterial-type PEPC in developing whole seeds during seed maturation from DS1 to DS7. D: The effect of nitrogen application on the expression of Gmppc2 protein. The measurement was duplicated, and the average of the values is shown. Error bar: Standard error (SE). Significance at *10% and **5% by Student's t-test. E: The effect of nitrogen application on PEPC activity. Error bar: SE.*

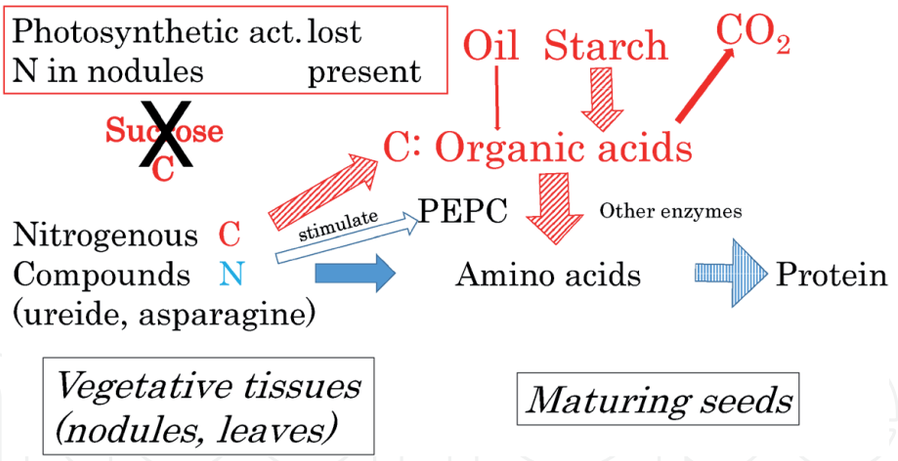

Figure 18.
Schematic presentation on the role of PEPC in the synthesis of storage protein on the late stage of seed growth maturation. Events in vegetative tissues and seeds at the late maturing stage of a CV, Enrei were drawn. Photosynthetic activity of leaves dramatically decrease by the decreases of N and chlorophyll contents, and resultantly supply of photosynthate (sucrose) to maturing seeds cease. On the other hand, nitrogenous compounds are supplied from degrading nodules to maturing seeds. Nitrogenous compounds are once separated into organic acids and amino residues in maturing seeds. Amino residues are introduced to newly synthesized organic acids of which carbon source is starch in maturing seeds to form amino acids which are substrates for the synthesis of storage protein. Carbon metabolizing enzymes including PEPC work to synthesize those organic acids. Activity of PEPC respond to the N supply to maturing seeds, thus affecting the protein content of seeds. Plant-type isozymes of PEPC work in the synthesis of amino acids through maturation period of seeds, an isozyme of Gmppc 2 was expressed at the late maturing stage of seeds.

exhibited a distinct expression pattern during soybean seed maturation. Namely, soybean seeds kept the expression of *Gmppc2* at a low level until the soybean seed is going to be matured (until DS4). In contrast, soybean seeds let the expression levels of *Gmppc2* at DS4 up-regulated drastically until seed maturation. Especially the expression level at DS6 in cotyledon was high. As mentioned in the previous section [3.3.1], DS6 is the critical stage at which seed protein accumulation varied between the two representative CVs [26]. To verify whether *Gmppc2* attributes to the different PEPC activity and protein content in the two CVs, expression of Gmppc2 protein was analyzed in soybean maturing seeds with the presence and absence of a nitrogen fertilizer, which suppressed nodulation and nitrogen fixation activity (**Figure 17**). The results indicated that soybean seeds highly expressed Gmppc2 protein at DS5 to DS7, and the expression was concordant with PEPC activity at DS5 and DS6 in response to the nitrogen fertilization. We illustrate the role of PEPC isozymes in the accumulation of protein (**Figure 18**). *Gmppc2* is the potential PEPC isogene, explaining the variation of seed protein content and PEPC activity among soybean CVs observed previously (**Figure 14**).

4. Conclusion: Our views of how nodules and their N_2 fixation activity affect protein and oil contents in soybean seeds

We carried out physiological experiments on the response of soybean plants to different types of N fertilizers. These experiments were designed from the viewpoint that there might be differences on the accumulation of storage protein and oil between soybean plants having different activities of N_2 fixation and N assimilation. We summarize our views on the characteristics of the accumulation of protein and oil in soybean seeds as follows.

1. Storage protein accumulation is independently regulated from storage oil accumulation in seeds.

We showed the accumulation profiles of protein and oil during seed maturation were quite different from each other (**Figures 9** and **10**). In immature seeds, PEPC plays a role in the protein accumulation (**Figure 16**), and its activity responds to the supply of N (**Figure 17**).

2. N_2 fixation results in decreasing the amount of oil per seed and leads to the decrease in oil content of seeds.

Seeds of nodulated CVs with high N_2 fixation activities have less oil content than those with low N_2 fixation activities (**Figure 4**; **Table 4**).

3. A high pseudo negative correlation between protein and oil was observed in seeds from plants which were grown on soil applied no N fertilizer.

High negative correlations were observed between contents of protein and oil in seeds of both nodulated and non-nodulated CVs grown under low N supply at the late maturation period of plants (**Figure 6**). Amounts of oil per seed were almost constant, and those of protein per seed were variable among plants in the cases of nodulated plants grown on L-L and non-nodulated ones grown on H-L (**Figure 7**). These results suggested that the observed high negative correlations between protein and oil contents in seeds were caused by the differences in the amount of accumulated protein in seeds among respective plants, and not related to oil accumulation in seeds.

4. Plants utilize soil N during the late growth stage as the source for seed protein, which implies the importance of N fertilization during the reproductive phase of soybean plants.

Based on the estimated amounts of fertilizer-derived N in seed protein, N excreted at the late period of plant growth was highly incorporated to seed protein in plants of both the nodulated and non-nodulated CVs (**Tables 2** and **3**). The higher seed yield of plants of regular nodulated CV grown on L-H plot than that grown on L-L and H-H plots suggested N supplied from both nodules and fertilizer at the late plant growth period increased seed yields (**Figure 2**).

5. Plant-type PEPC gene family plays a role in the synthesis of amino acids for storage protein. Gmppc2 isogene may have a crucial role in accumulating protein on the late maturation period of seeds.

Pattern analysis suggested that PEPC promotes protein accumulation but not oil accumulations (**Figure 16**). As the increase of acetyl Co-A carboxylase activity in immature rapeseed by gene engineering increased seed oil content [29], this enzyme might play a key role in oil accumulation in soybean seeds. These enzymes might work to synthesize respective storage compounds, protein and oil, independently with each other. Plant-type PEPC isogenes were expressed in immature seeds through the seed maturation period (**Figure 17C**). *Gmppc2*, an isogene of plant-type PEPC, was expressed in seeds at the late maturation period, and its PEPC protein expression level was higher in seeds of low N condition than in those grown on high N one (**Figure 17D**). These observations coincides with the observations that seeds of low N soil (L-L plot) had higher protein content than those grown on high N soil (H-H plot) (**Figures 3** and **6**).

We observed that the ratio between changing amounts of protein and oil in seeds was almost the same among plants grown on soils fertilized with different types of CUSLNFs (**Figure 7**). This observation implied that the allocation ratio of C to protein and oil was controlled by some mechanism.

Genetic studies on the storage compounds of soybean seeds have made progress. A quantitative trait locus analysis mapped regions controlling the contents

of storage compounds in soybean seeds on the contents of protein and oil on the soybean genome [30]. Concerts between the physiological and genetic approaches are useful to elucidate the mechanism of how contents of storage compounds are controlled in soybean seeds.

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