

Seed Storage Proteins; Strategies for Developing Crops Promoting Human Health

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1. Introduction

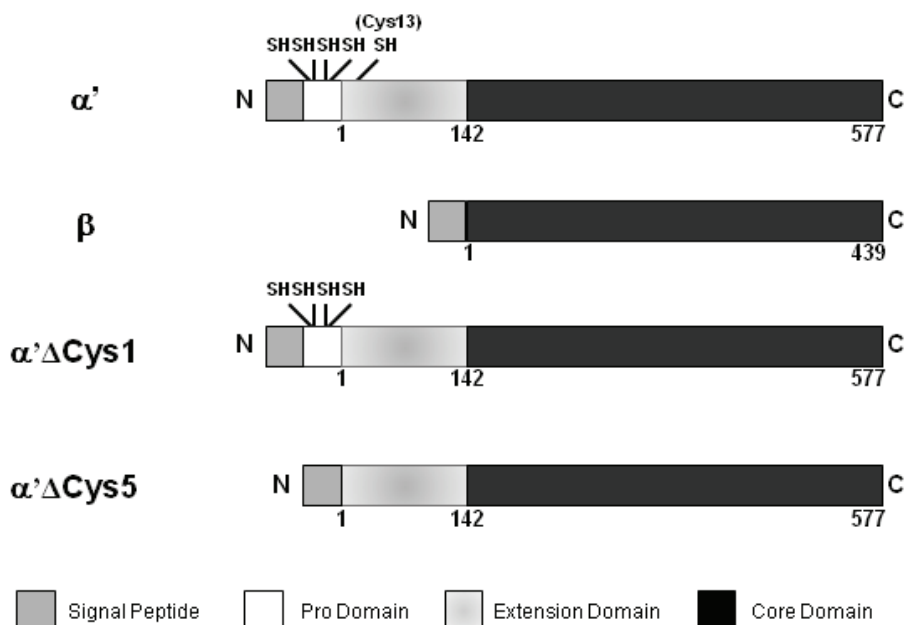
Plant seeds contain high amounts of storage proteins. These are classified on basis of their solubility as water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and acid- or alkaline-soluble glutelins (Osborne 1924, Utsumi, 1992). The compositions of seed storage proteins differ among plant species. For examples, monocot seeds contain mainly glutelins and prolamins (Ogawa et al., 1987; Li & Okita, 1993; Cagampang et al., 1966), whereas legume seeds contain mainly 7S/11S globulins (Utsumi, 1992).

Rice is the staple food of approximately half of the population of the world. The major seed storage proteins of rice are glutelins and prolamin, similarly to the other monocots. The seed storage proteins present in rice, however, offer little significant benefit to human physiology. Therefore, improving the nutritional and physiological values of rice would be of benefit to the health of considerable numbers of people. A candidate protein that might be of interest in the context of improving the physiological values of rice is β -conglycinin. The seed storage protein of soybean, β -conglycinin, lowers plasma cholesterol and triglyceride levels in humans (Sirtori et al., 1995; Aoyama et al., 2001). Moreover, β -conglycinin increases adiponectin levels and improves glucose tolerance (Tachibana et al., 2010). The α' subunit of β -conglycinin has LDL-cholesterol-lowering activity (Sirtori and Lovati, 2001) and contains a phagocytosis-stimulating peptide (Tsuruki et al., 2003). Therefore, development of rice that can accumulate β -conglycinin should produce a staple food with several important physiological benefits to human health.

β -Conglycinin has the trimeric structure common to 7S globulins of other plant species and is composed of three subunits, α , α' and β . The α and α' subunits contain an N-terminal extension in addition to a core region common to all the subunits (Maruyama et al., 1998, 2001 & 2004). The β subunit consists of only the core domain. The α and α' subunits and the β subunit are synthesized on polysomes as prepro- and pre-forms, respectively. The signal peptides are co-translationally removed, the polypeptides are N-glycosylated with high-mannose glycans and assemble into trimers in the ER (Yamauchi & Yamagishi, 1979; Utsumi, 1992). They are transported from the ER to the protein storage vacuoles through the Golgi apparatus (Mori et al., 2004). The pro regions of the α and α' subunits are

proteolytically processed to give their mature forms, but processing enzymes are unknown. Both the α and α' subunits contain four cysteine (Cys) residues in their pro regions and one Cys in the mature extension region (Figure 1).

We expressed the α' and β subunits of soybean β -conglycinin in rice to develop a line with the potential to promote human health. The accumulation behavior of β -conglycinin in rice seeds has also been described. Further, we designed a β -conglycinin molecule by protein engineering that is expected to have enhanced physiological functions with regard to promoting human health. We expressed this construct in rice plants. In this chapter, we describe our strategy to develop a novel crop by introduction of soybean seed storage protein.



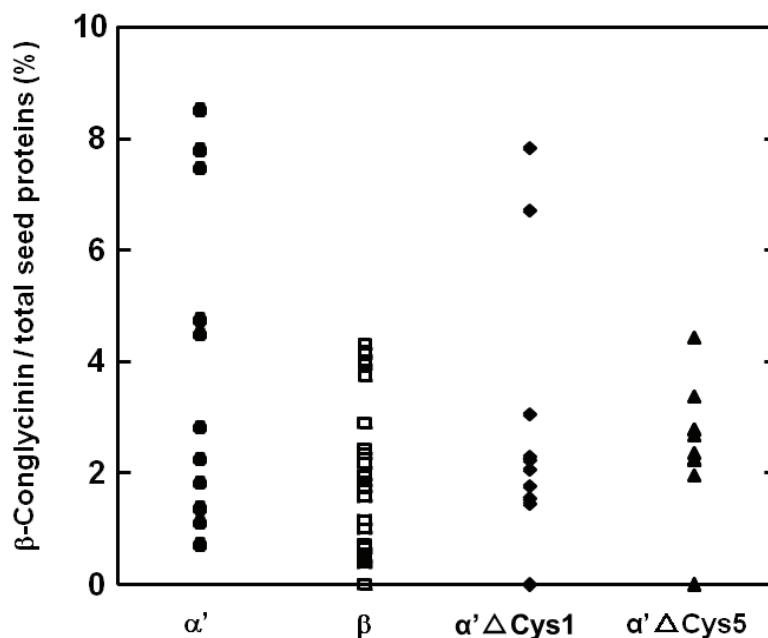
α' , wild type α' subunit; β , wild type β subunit; $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$, modified versions of the α' subunit. SH indicate positions of cysteine residues. This figure is modified from Motoyama et al. (2009) with permission

Fig. 1. Schematic presentation of the structure of wild-type and mutated β -conglycinin subunits.

2. Transgenic rice producing β -conglycinin in seeds

2.1 Development of transgenic rice seeds accumulating α' and β subunits

cDNAs of the α' and β subunits driven by the rice glutelin *GluB-1* and *GluB-2* promoters, respectively (Takaiwa et al., 1996), were introduced into rice calli by *Agrobacterium tumefaciens*-mediated transformation (Goto et al., 1999). The levels of α' and β subunits in total seed protein extracts were estimated immunologically and found to average levels of 3.9% and 2.0%, respectively (Figure 2). The difference in levels of the two subunits was statistically significant.



Total protein was extracted from each of the transgenic seeds with SDS buffer. Aliquots (1 μ g/1 μ l) were spotted on a nitrocellulose membrane and the recombinant proteins were detected immunologically with either anti- α' or anti- β sera. Accumulation levels of recombinant proteins were expressed as a percentage of total seed protein. Each mark represents the accumulation level in an independent transgenic plant. This figure is reprinted from Motoyama et al. (2009) with permission.

Fig. 2. Comparison of the accumulation levels of β -conglycinin in transgenic rice seeds.

2.2 Transcription levels of α' and β subunits in rice seeds

The rice lines that exhibited the highest levels of the subunits were self-pollinated to obtain homozygous lines. The α' and β subunits represented 7.9 ± 0.7 and $4.4 \pm 0.8\%$, respectively, of the total rice seed protein extract. Again, the α' subunit accumulated at about twice the rate of the β subunit, similarly to the T_1 seeds. We compared the levels of mRNA of the α' and β subunits by real-time PCR to examine the relationship between mRNA and protein levels. Total RNAs from seeds at 15 days after flowering in the homozygous lines were analyzed and the transcription level of the α' subunit (line 6-2) found to be similar to that of the β subunit (line 5-4), although the α' subunit protein accumulated at about twice the rate as that of the β subunit. Thus, differences in the transcription do not underline the difference in the rate of accumulation of the proteins in rice seeds.

2.3 Post-translational modification of α' and β subunits in rice seeds

Both the α' and β subunits are N-glycosylated with high-mannose type glycans in soybean seeds (Yamauchi & Yamagishi, 1979). To examine whether the α' and β subunits synthesized in rice seeds are also N-glycosylated, the subunits were digested with either PNGase F or Endo H. PNGase F hydrolyzes almost all N-glycans, excluding the core-fucosylated complex

N-glycan, while Endo H primarily hydrolyzes high-mannose glycan but not complex glycan. Prior to the digestions, no degradation products of either the α' or β subunits could be detected by western blotting. Thus, both the α' and β subunits accumulated in a stable fashion in rice seeds. After digestion with the appropriate enzyme, each subunit produced a single band of a lower molecular mass than that of the intact subunit. The analysis suggests that both the α' and β subunits are glycosylated with high-mannose N-glycan, and not with complex glycan.

2.4 Interaction of the α' and β subunits with rice seed storage proteins

We performed a sequential extraction of seed proteins from transgenic seeds using buffer (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.02% (w/v) NaN_3) without 2-mercaptoethanol, lactic acid and SDS buffer. Generally, rice glutelin, one of the major types of seed storage proteins in rice, can be extracted by lactic acid but not by buffer (Tanaka et al., 1980; Katsube et al., 1999). We found that most of the β subunit was extracted by the buffer without 2-mercaptoethanol. In contrast, a large amount of the α' subunit was extracted by the lactic acid in addition to the buffer without 2-mercaptoethanol. The α' subunit extracted by the lactic acid exhibited several bands on an SDS-PAGE gel in the absence of 2-mercaptoethanol, whereas, in its presence, only one band appeared. We subjected this fraction to two-dimensional electrophoresis (without/with 2-mercaptoethanol). Most of the stained proteins in the first dimension were identified as glutelins in the second dimension, while the α' subunit exhibited several bands. The major band of the α' subunit might be derived from a complex formed by the α' subunit and rice acid-soluble proteins in first dimension. Other bands of the α' subunit were also detected in the high-molecular mass region. Glutelins were detected in a region of molecular mass higher than the monomer of the α' subunit. These results suggest that some of the α' subunit forms one or more disulfide bonds with glutelin.

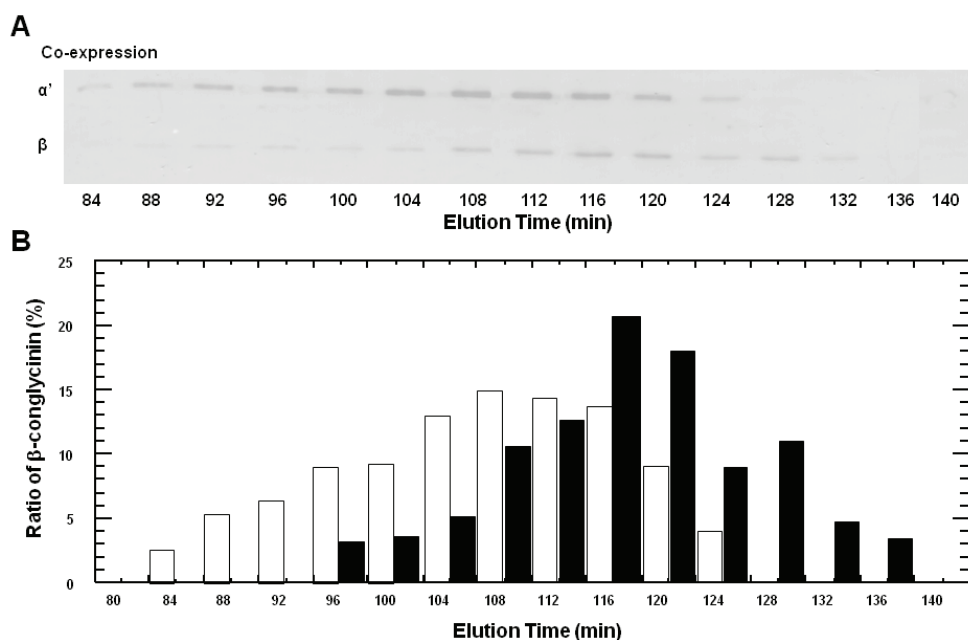
2.5 Role of cysteine residues in the α' subunit

To study the role of Cys residues of the α' subunit (four residues in the pro-region and one residue in the mature subunit) in the accumulation of the subunit in rice seeds, we developed transgenic rice producing $\alpha'\Delta\text{Cys1}$ or $\alpha'\Delta\text{Cys5}$ (Figure 1). On average, $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ comprised 3.2 and 2.5%, respectively, of total rice seed proteins (Figure 2). More than 90 % of the total $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ of transgenic rice seeds could be extracted with the buffer without 2-mercaptoethanol, similar to the β subunit. Further, $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ formed the expected assembly in transgenic rice seeds. Therefore, the higher levels of accumulation of the α' subunit compared to the β subunit might not be due to a disulfide bond interactions with glutelin.

2.6 Transgenic rice crosses producing both the α' and β subunits in seeds

Transgenic rice seeds with seeds exhibiting high levels of the α' and β subunits were selected to develop transgenic lines with increased accumulation of both subunits. Total proteins of F1 seeds were extracted with SDS buffer. Transgenic rice producing only the α' subunit, or the β subunit, or both α' and β subunits were identified, and non-transgenic rice was also obtained. The levels of the α' and β subunits in the co-expression lines were compared with those in a single expression line. In co-expression lines, the level of the α'

subunit was about 15% lower than that in the transgenic rice producing only the α' subunit. By contrast, the level of the β subunit in the co-expression lines was about 60% higher than in the transgenic rice producing only the β subunit. A homozygous transgenic rice line that produced both the α' and β subunits was obtained and used for further analysis. In this line, the levels of the α' subunit were approximately 5.2 % and 5.4%, respectively, of those in transgenic rice producing only the α' subunit or producing both α' and β subunits. Similarly, the levels of the β subunit were approximately 3.3% and 5.5%, respectively, of those in transgenic rice producing only the β subunit or both α' and β subunits. The overall level of the α' and the β subunits in transgenic rice producing both the α' and β subunits was approximately 10%. Therefore, co-expression of the α' and β subunits increased the rate of accumulation of β -conglycinin as compared to transgenic rice seeds producing a single subunit of β -conglycinin.



A; The α' and β subunits were extracted with a buffer (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.02% (w/v) NaN_3) with 2-mercaptoethanol from rice seeds co-expressing both subunits and subjected to Sephacryl S-300 HR column. Fractions were collected every 4 min. The fractions from 84 to 140 min were subjected to SDS-PAGE followed by western blotting.

B; Summary of the results of western blot analysis of seeds of rice co-expressing both subunits. White and black bars indicate the α' and β subunits, respectively. The figure is reprinted from Motoyama et al. (2010a) with permission.

Fig. 3. Analysis of molecular assembly of the α' and β subunits by gel filtration column chromatography

Soybean seeds contain three subunits of β -conglycinin that form homo- and hetero-trimers in random combinations (Maruyama et al. 2002a and 2002b). To investigate whether the α' and β subunits of transgenic rice assembled into heterotrimers, extracts from seeds obtained

using buffer with 2-mercaptoethanol was analyzed by the gel filtration chromatography (Figure 3). In transgenic rice producing only the α' subunit, homo-trimeric α' subunit was detected in eluates between 96 and 112 min. While a homotrimeric β subunit was detected in eluates between 124 and 136 min in transgenic rice producing only the β subunit. By contrast, the α' subunit in transgenic rice producing the α' and β subunits was detected from 84 to 124 min with peaks at 108 and 112 min and the β subunit was detected in eluates from 96 to 136 min with a peak at 116 min. The α' and β subunits in transgenic rice seeds producing both the α' and β subunits exhibited wider ranges of elution compared to transgenic rice producing a single subunit. This indicates that heterotrimers composed of the α' and β subunits are present in the transgenic rice producing both the α' and β subunits. The α' and β subunits in transgenic rice producing both the α' and β subunits were sequentially extracted from transgenic rice seeds by buffer without 2-mercaptoethanol, 1% lactic acid and SDS buffer. The lactic acid extraction solubilizes glutelins and the α' subunit linked to glutelin. Three intense bands corresponding to the α' and β subunits were observed in the extract of the buffer without 2-mercaptoethanol. Multiple bands corresponding to the α' subunit were detected in the extract with the lactic acid. An interaction between rice acid soluble proteins (mainly glutelin) and the α' subunit via a disulfide bond might occur in transgenic rice producing the α' and β subunits, similar to that observed in plants producing only the α' subunit.

2.7 Subcellular localization of β -conglycinins in rice seeds

Rice seeds have two types of protein bodies, termed PB-I and PB-II (Oparka & Harris, 1982; Tanaka et al., 1980; Yamagata, & Tanaka, 1986; Krishnan & White, 1995). PB-I are derived from the ER, PB-II from the vacuole. β -Conglycinin is known to accumulate in the vacuole (Mori et al., 2004). The subcellular distributions of the α' subunit, the β subunit, $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ in transgenic rice seeds were analyzed by transmission electron microscopy. PB-II showed a uniform electron density in non-transgenic seeds. However, in mature seeds of transgenic rice producing the α' subunit, the electron density of the entire PB-II was high and the α' subunit was detected only in the peripheral region of PB-II. By contrast, regions of low electron density regions in the PB-II of mature seeds of transgenic rice producing the β subunit; the β subunit was localized in these regions. In mature seeds of transgenic rice producing $\alpha'\Delta\text{Cys1}$ or $\alpha'\Delta\text{Cys5}$, low density regions were formed in PB-II similarly to transgenic rice producing the β subunit. $\alpha'\Delta\text{Cys5}$ was located only in the low density regions, whereas $\alpha'\Delta\text{Cys1}$ was found in both low- and high-density regions. These results indicate that the pro region of the α' subunit affects protein distribution within PB-II.

We compared the distributions of the α' and β subunits with glutelin in a developing seeds. Glutelin was located in the PB-II of transgenic rice seeds producing the α' subunit and was co-localized with the α' subunit in the periphery of the PB-II. Regions of low and high electron density were observed in the developing seeds of the transgenic rice that produced the β subunit, similar to those observed in the mature seeds. Glutelin did not localize with the β subunit in the PB-II. The patterns of distribution of $\alpha'\Delta\text{Cys1}$ and $\alpha'\Delta\text{Cys5}$ were similar to those in mature seeds. Glutelin was observed in the high electron density regions, and not the low electron density regions, in transgenic rice seeds producing $\alpha'\Delta\text{Cys1}$ or $\alpha'\Delta\text{Cys5}$. These results, together those of the sequential extraction experiment, suggest that the α' subunit might interact with glutelin via the pro region in transgenic rice seeds and that this interaction plays an important role on the localization of the α' subunit within the PB-II.

In addition, we examined the accumulation of α' and β subunits in developing seeds of transgenic rice producing both subunits. In seeds at 10 days after flowering, a low electron density region in PB-II was observed. The α' and β subunits were both present in the low electron density regions, although the α' subunit was located outside of this region. In contrast, the β subunit did not localize outside of the low electron density region of the PB-II. It is possible that homotrimers of the α' subunit in seeds of transgenic rice producing both the α' and β subunits might accumulate outside of the low electron density region of the PB-II.

3. Transgenic rice with a high phagocytosis stimulating activity

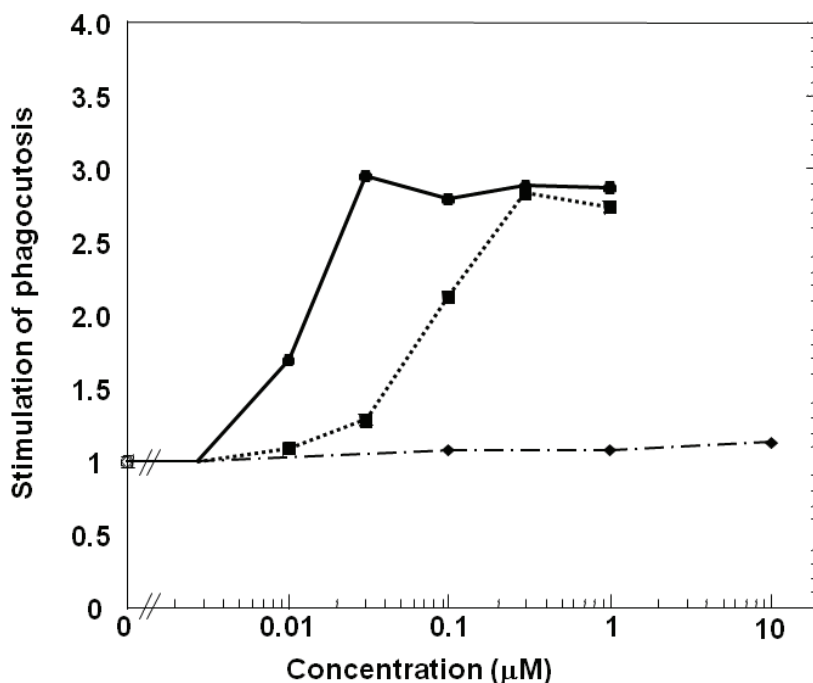
3.1 Design of β subunit with a high phagocytosis stimulating activity

A phagocytosis-stimulating peptide (MITLAIPVKNKPGR) was isolated from trypsin digested soybean proteins and named soymetide-13 (Tsuruki et al., 2003). Soymetide-13 corresponds with a fragment of the α' subunit of β -conglycinin. Although the N-terminus of soymetide-13 is not formylated, it acts as an agonist of the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor present on the surfaces of neutrophils and macrophages (Tsuruki et al., 2003; Williams et al., 1977). fMLP is strongly chemotactic for neutrophils (Showell et al., 1976). The fMLP receptor stimulates phagocytosis and mediates the generation of reactive oxygen species in neutrophils and macrophages (Tsuruki et al., 2003). Replacement of the third residue from the N-terminus of soymetide-13 with Trp ([Trp³]-soymetide-13) resulted in a significant increase in affinity for the fMLP receptor (Tsuruki et al., 2004). On the other hand, the β subunit of β -conglycinin has an analogous sequence (IIKLAIPVKNKPGR) to soymetide-13. However, it lacks phagocytosis-stimulating activity, because its N-terminus is not methylated. To introduce the phagocytosis activity into the β subunit, we replaced residues I122 and K124 in the analogous sequence of the β subunit (IIKLAIPVKNKPGR) and designed three mutants, I122M/K124T, I122M/K124F and I122M/K124W (Maruyama et al., 2003). First, we constructed model simulations of the three mutants from the three dimensional structure of the β subunit. Root mean square deviation for all the C α atoms in a monomer between the starting and simulated models was around 0.67 Å for all the mutants and the distances of C α atoms between the wild type and the simulated models at positions 122 and 124 were 0.47-0.49 and 0.29-0.50 Å, respectively, in all mutants. These values suggest that all the mutants could fold correctly. To confirm this conclusion, we characterized the structural features of the mutants after expression in *E. coli*. No significant differences in circular dichroism spectra were observed between the wild type and the mutants. Measurement of T_m values by differential scanning calorimetry yield values for the mutants that were 1.9-3.1 °C lower than the wild type. Although there is a hydrogen bond between Lys124 and Tyr109 in the β barrel of the wild type, all of the mutants lost this hydrogen bond by the replacement of Lys124. Therefore, the loss of the hydrogen bond of the mutants might induce the slight decrease in T_m values. In gel filtration chromatography, all of the mutants eluted similarly to the wild type. We also determined the crystal structure of I122M/K124W to investigate the effect of the induced change in detail, since Trp was largest of the introduced residues. The C α distances at the residues 122 and 124 between the wild type and I122M/K124W were 0.48 Å and 0.17 Å, respectively. No unfavorable van der Waals interactions were found between the side chains of the replaced residues and neighboring residues. These results indicate that the replacement had little influence on backbone structures and that our conclusions on the conformation of I122M/K124W from

the simulated model are correct. Further, all of the mutants exhibited phagocytosis-stimulating activity in the order of I122M/K124T < I122M/K124F < I122M/K124W as expected, whereas the wild type did not. These results indicate that I122M/K124W has a higher phagocytosis stimulating activity than the α' subunit.

3.2 Development of transgenic rice with a high phagocytosis stimulating activity

To develop a rice line with high phagocytosis stimulating activity, the cDNA for I122M/K124W driven by the rice glutelin *GluB-2* promoter was introduced into the rice genome. The highest level of accumulation of I122M/K124W was 4.1 % of total rice seed proteins, a level similar to that of the β subunit in transgenic rice (Motoyama et al., 2010b). The I122M/K124W was extracted in a salt-soluble fraction from transgenic rice seeds in a similar fashion to the β subunit. An electron microscopic analysis showed that I122M/K124W was located in a low electron density region in the PB-II of mature transgenic rice seeds. The β subunit was also localized to these regions, as described above. These observations indicate that the modification of the β subunit did not affect accumulation or localization in rice seeds.



The solid line represents I122M/K124W purified from transgenic rice; the dotted line represents I122M/K124W purified from *E. coli*; the dashed and dotted line represents the wild type of the β subunit purified from transgenic rice. The horizontal axis indicates the final concentration of the wild-type and mutated β subunits in the phagocytosis assay. Protein concentration values before trypsin digestion were used to produce the plot. The figure is reprinted from Motoyama et al. (2010b) with permission.

Fig. 5. Comparison of phagocytosis-stimulating activity

To investigate whether the I122M/K124W can assemble into a trimer in rice seeds, as the β subunit does in soybean seeds, we extracted and purified the mutant protein from transgenic rice seeds and subjected it to gel filtration chromatography. The I122M/K124W peak eluted at a similar time as the homotrimer of the β subunit prepared from soybean seeds and from transgenic rice seeds producing the wild type of the β subunit. Purified I122M/K124W was digested by PNGase F and/or Endo H. After digestion, the I122M/K124W yielded a single band on SDS-PAGE with a molecular mass lower than that of the intact subunit.

The difference in mobility between glycosylated and non-glycosylated β subunits on SDS-PAGE is consistent with results of the wild type (Motoyama et al., 2009). Our analyses indicate that the I122M/K124W in the transgenic rice seed folds correctly, assembles into a trimer, and is modified by attachment of N-linked glycans similarly to the β subunit in soybean seeds.

Trypsin-digested peptides from wild-type and I122M/K124W produced in transgenic rice were assayed for their effect on phagocytosis activity. In addition, we produced I122M/K124W produced in *E. coli* was used for comparison to that purified from the transgenic rice seeds. The rice-derived I122M/K124W exhibited a high phagocytosis-stimulating activity, whereas the wild-type of β subunit purified from transgenic rice seeds had a barely detectable level of activity (Figure 5). Moreover, the phagocytosis-stimulating activity of the rice-derived I122M/K124W was higher than from *E. coli*. We used trypsin digestion and subsequent HPLC analysis to analyze the yield of [Trp³]-soymetide-13 from I122M/K124W produced in rice seeds and in *E. coli*. The yield of [Trp³]-soymetide-13 by the rice-derived protein was estimated as 35.2 %, while that from *E. coli* was 7.7 %. The presence of the glycan on rice-derived I122M/K124W produced a higher solubility at neutral pH compared to that produced in *E. coli*. This might be due to a relatively higher yield of the phagocytosis-stimulating peptide, thereby causing apparent increase in its activity.

4. Conclusion

It has been reported that β -conglycinin has many physiological functions. Plasma cholesterol and triglyceride levels are decreased in rats fed 20 mg/(kg body weight)/day of the α' subunit (Duranti et al., 2004). This is the equivalent of 1.2 g/(60kg body weight)/day of α' subunit in humans. The maximum accumulation level of the α' subunit was about 8% of total seed protein, and the rice seed proteins account for 7% of the total dry weight of rice seed. The average daily consumption of rice in Japan is 150g, which will therefore contain about 0.84 g of the α' subunit. To increase the levels of the α' subunit to provide a greater physiological effect, then an increase by a factor of 1.5 would be necessary. It was reported that a mutant rice variety with low seed storage protein mutant variety is a good platform for the production of foreign proteins (Tada et al., 2003; Wakasa et al., 2007). We plan to utilize a rice variety lacking some glutelin subunits, but with a delicious taste, to develop rice producing β -conglycinin at a high amount. Moreover, multiple introductions of bioactive peptide into β -conglycinin by protein engineering can fortify physiological values. In the future, this strategy could be used to develop transgenic rice that can prevent lifestyle-related diseases and promote a human health in developed countries.

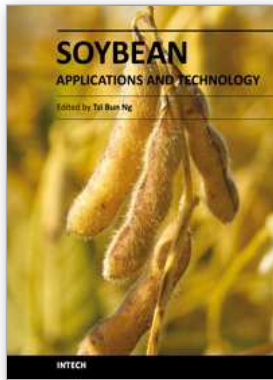
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