

称号及び氏名	博士 (応用生命科学)	Guray Akdogan
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論文名	Effects of double mutation of the <i>waxy</i> and <i>amylose-extender</i> genes on gene expression profiles and metabolites in rice (イネにおける <i>waxy</i> と <i>amylose-extender</i> 二重変異の遺伝子発現と代謝産物への影響)	
論文審査委員	主査	北村 進一
	副査	乾 隆
	副査	乾 博
	副査	杉本 憲治

論文要旨

Introduction

Rice (*Oryza sativa*) is the staple food for billions of people. In addition to its agronomic importance, rice is also an important biological model species for monocot plants. The International Rice Genome Sequencing Project has finished sequencing the rice genome. To date, more than 30,000 full-length cDNA sequences of *Oryza sativa* have been deposited in a publicly available database. This database offers a complete overview to understand the biological functions of the genes. At this time, gene expression analysis comprises one of the most important areas of gene function studies.

Starch is the reserved carbohydrate form produced in the cereal's endosperm. Starch granules consist of two types of glucose polymers: mainly linear amylose in which D-glucosyl units are linked with α -1,4 glucosidic bonds and a few branches of α -1,6 linkages, and amylopectin which is a highly branched molecule consisting of short amylose chains connected with α -1,6 linkages. Starch is synthesized in the amyloplast of the endosperm cells by the coordinated action of a group of enzymes. Amylose is synthesized by ADP glucose pyrophosphorylase (AGPase) and granule-bound starch synthase (GBSSI), while amylopectin is synthesized by AGPase, starch synthase (SS), starch branching enzyme (SBE) and starch debranching enzyme (DBE). Mutations in the genes involved in starch synthesis result in not only altered starch structures, but also changes in grain composition and gene expression profile. Waxy (wx) protein, encoded by *GBSSI*, is responsible for amylose synthesis in the rice endosperm. The loss of GBSSI activity by mutation results in amylose-free (*waxy*, *wx*) starch phenotypes. Starch-branching enzymes (SBE) play an essential role in the

synthesis of amylopectin. Mutation in the gene for SBEIIb results in high amylose (*amylose extender, ae*) starch phenotypes.

A series of studies were conducted on the effect of the loss of activity of GBSSI and SBEIIb on amylopectin fine structure and gene expression levels in the developing rice endosperm.

Chapter 1. Verification of mutants and determination of grain composition

1.1. Verification of mutants

A crude extract from kernels harvested at 15 days after flowering was subjected to immunoblot and zymogram analyses to confirm the presence of defective enzymes by mutation. An immuno-blot analysis using antiserum raised against rice granule-bound starch synthase I (GBSSI) showed that *wx* and *wx ae* were completely defective in WX (GBSSI) protein. A starch zymogram analysis showed that branching enzyme IIb (SBEIIb) activity was defective in *wx ae* when the native gel was incubated with glucose-1-phosphate and rabbit phosphorylase. An immuno-blot analysis using antiserum raised against rice SBEIIb showed that SBEIIb was not detected in the crude extract from a *wx ae* kernel. Each developing mutant endosperm stained the same color as its mature endosperm, and the starch of each mutant endosperm did not change phenotype between 15 DAP and maturity.

1.2. Determination of grain composition

The grain compositions of *wx*, *wx ae* and wild type were investigated in immature and mature grains. *wx* and wild type are similar in seed weight and composition, while *wx ae* is significantly different. The grain weight of *wx ae* was 10% less than those of wild type and *wx* at 15 DAP and 32% less at maturity. The amount of accumulated starch between 15 DAP and maturity increased 5.2 and 4.6 mg/grain in *wx* and wild type, respectively, but only 1.1 mg/grain in *wx ae*, showing clearly that starch synthesis in *wx ae* was interrupted. The amount of accumulated sucrose in *wx ae* was more than twice the level found in *wx* and wild type both at 15 DAP and at maturity.

Chapter 2. Structure, physical and digestive properties of starch of the mutant endosperms

After iodine staining, the starch from the endosperms of mutants clearly showed a different color from that of the wild type. The *waxy (wx)* starch had a lower affinity for iodine because it lacks amylose. The *amylose extender (ae)* was named for the apparently higher amylose content calculated on the basis of the blue value of the starch on staining with an iodine solution. The blue value was not dependent on the presence of amylose, but on longer chains in amylopectin produced as a result of a defect in BEIIb. In this study, even though the mutant contained no amylose, the endosperm of the double mutant of *wx* and *ae* (*wx ae*) stained purple.

The *wx ae* and *ae* starches showed no significant difference in the chain-length distribution of amylopectin and starch granule morphology. The *wx ae* starch displayed a higher pasting temperature and higher peak viscosity. The gelatinization peak temperatures of the *wx*, *ae*, and *wx ae* starches were 2.7°C, 13.6°C, and 17.7°C higher respectively than that of the wild-type starch, and the *wx ae* starch showed a retrogradation peak with a shorter cooling period than that of *ae*. The raw *ae* and *wx ae* starches were almost indigestible by α -amylase *in vitro*. Rats fed the *wx ae* starch showed slowly increasing blood glucose at a lower level than the rats fed the *wx* or wild-type starch. These results indicate that the primary structure of the rice *wx ae* amylopectin with enriched long chains changes the granular structure of the starch including its crystal structure and results in resistance to *in vitro* and *in vivo* degradation.

Chapter 3. Transcriptome analysis

3.1. Effects of mutation on gene expression profiles related to carbohydrate metabolism.

Starch biosynthesis is important during endosperm development. Much is known about the regulation of gene expression involved in starch synthesis; less information is available on the changes in genome-wide expression profiles as a consequence of impaired starch synthesis. I used the High-Coverage Gene Expression Profiling (HiCEP) method to analyze transcription profiles of the genes expressed in the *wx* mutant, *wx ae* double-mutant and wild type rice endosperms at the mid-grain filling stage. To construct the template for HiCEP analysis, total RNA was extracted from seeds harvested at 15 days after pollination.

The coverage ratio of the HiCEP method was estimated by constructing a template *in silico* using a full-length cDNA sequence extracted from the KOME database. I concluded that 70% (21,439) of rice transcripts can be analyzed by using HiCEP. I identified 13,046 validated peaks corresponding to single transcripts in all three samples.

Among 13,046 peaks detected by HiCEP, 11 and 140 genes were up-regulated more than 2-fold the level of wild type in *wx* and *wx ae*, respectively, while 21 and 226 genes were down-regulated to less than one-half the level of wild type. The expression level of sucrose transporter 1 (*SUT1*), invertase a (*INVa*) and sucrose synthase 3 (*SuSy3*), which are involved in sucrose metabolism, significantly decreased in *wx ae* compared to *wx* and wild type. The expression level of genes involved in starch synthesis did not change significantly in *wx* or *wx ae* except for *SSIIIa* which was down-regulated in *wx ae*. However, *AAmy1A*, *AAmy3E*, *PHoH* and *DPE2* which are involved in starch degradation and modification were up-regulated in *wx ae*. Functional classification of the genes differentially expressed in *wx ae* showed that they were involved in macromolecule, cellular and primary metabolic processes or were stress-related.

The results given in Chapter 1 clearly show that the sucrose transported from source organ to sink organ cannot be converted to starch efficiently in *wx ae*. Reduced starch synthesis and high sucrose concentration in *wx ae* cause pleiotropic effects and transcriptional changes for a number of genes. In *wx ae*, a number of differentially expressed genes involved in lipid and secondary metabolite metabolic processes were also identified. The excess sugar in *wx ae* might be used to synthesize alternative storage compounds such as secondary metabolites or fatty acids.

3.2. Effects of mutation on gene expression profiles related to osmotic stress

In the *wx ae* double mutant, reduced starch synthesis results in sucrose accumulation in the developing endosperm. Sugars have essential roles as substrates in carbon and energy metabolism and in polymer synthesis. Sugars also have important hormone-like functions as primary messengers in signal transduction and transcript stability. I identified 131 transcripts which are stress-related genes. Among these genes, the expression levels of abscisic stress ripening protein 1, serine/threonine-protein kinase OSR1 (Oxidative-stress responsive 1 protein) and 22.7 kDa class IV heat shock protein, which are related to osmotic stress, were 12, 5 and 2 times higher in *wx ae* than in wild type, respectively. The expression level of *AAmy* was up-regulated in *wx ae*. Changes in *AAmy* expression level were previously reported in plant growth under stress conditions. It is also reported that the expression level of the seed storage proteins 13 kDa prolamin and preproglutelin are down-regulated under stress conditions.

The changes in composition of the *wx ae* double mutant grain, particularly the storage of large amounts of osmotically active sugars, could potentially result in changes in osmotic pressure and water potential in the

grain. This increase of sugars may be the result of increased synthesis of proteins involved in stress responsiveness.

Chapter 4. Expression and characterization of rice disproportionating enzymes

This chapter gives a characterization of disproportionating enzyme (DPE1) and its isoform DPE2 in rice, since the *OsDPE2* gene is found up-regulated by the double mutation of GBSSI and SBEIIb. Rice *DPE* genes (*OsDPE1* and *OsDPE2*) were cloned and expressed in *E. coli*. *OsDPE1* and *OsDPE2* genes encode proteins of 594 and 946 amino acids with a calculated molecular mass of 67 kDa and 108 kDa, respectively. Purified recombinant OsDPE1 and OsDPE2 showed highest activity at around pH 7.0 and pH 6.0 – 7.0, respectively. The optimum reaction temperature was 30°C for OsDPE1 and 39°C for OsDPE2. Recombinant OsDPE1 disproportionates maltotriose to produce glucose and maltopentaose, and thus shares the defining behavior of D-enzymes. In our experiments, recombinant OsDPE2 catalyzed the glucose transfer reaction from maltose to an acceptor molecule such as glycogen. I also characterize the differences between the diurnal transcription profiles of *OsDPE1* and *OsDPE2* in rice leaves and seeds, and their temporal expression levels in developing rice seeds.

Conclusion

The structure of amylopectin altered by *wx*, *ae* and *wx ae* mutations in rice was determined and its relationship to the gelatinization, retrogradation and digestibility of rice starches *in vitro* and *in vivo* was clarified. Changes in expression level of the genes expressed in the *wx* mutant and *wx ae* double-mutant were determined using an improved amplified fragment length polymorphism technique. The *wx ae* double mutations caused pleiotropic effects and transcriptional changes for a number of genes involved in sucrose metabolism, starch synthesis and starch degradation and modification. One of the up-regulated genes in *wx ae*, disproportionating enzyme 2 (DPE2), and its plastidial isoform disproportionating enzyme 1 (DPE1) were cloned and characterized.

審査結果の要旨

イネは、胚乳部分であるコメが多くので食されている重要な栽培植物である。また、単子葉植物のモデル植物としても重要で、そのゲノムの全配列が公表されている。3万以上の完全長 cDNA の配列情報がデータベースとして提供され、遺伝子の発現を網羅的に解析するのに利用されている。

コメの主要な成分はデンプンであり、デンプンの改変を目的とした、デンプン生合成酵素遺伝子の突然変異体についてはこれまでも多く研究されてきた。しかしながら、デンプン生合成酵素遺伝子の改変と酵素活性の喪失が胚乳内の全遺伝子の発現と代謝産物にどのように影響を及ぼすかについては不明な点が多いのが現状である。学位申請者はデンプンの合成にかかわる二つの遺伝子、すなわち *waxy(wx)*と *amylose-extender(ae)*遺伝子にストップコドンが挿入され、*granule-bound starch synthase (GBSSI)*と *starch branching enzyme(SBEIIb)*の活性が失われた二重変異体を対象にして、そのデンプンの構造を明らかにするとともに、その変異がどのように胚乳の遺伝子発現と代謝産物に影響を及ぼしているかについて研究を行った。

第1章では、開花後15日目のイネ種子試料を用いて、*wx ae* 二重変異体での GBSSI と SBEIIb 酵素タンパクの喪失を確認するとともに、炭水化物などの構成成分の変化について明らかにしている。デンプンの合成量は *wx ae* 変異体では本品種の金南風(WT)に比較して 22% 減少していた。水溶性のグルカンの含有量は約 43%の減少が認められた。グルコース、フルクトースでは変化は観察されず、マルトースの含有量は 32%減少していた。一方、スクロースの含有量は約2倍増加していた。また、興味深いことに総脂質が約2倍増加しており、その中に溶解していると考えられるビタミンEや総オリザノール量も約2倍増加していた。デンプンの生合成の原料となるスクロースがデンプン合成に有効に使われず、脂質の生合成に使われたものと考察された。

第2章では、デンプンの構造と物性に及ぼす変異の影響を明らかにしている。GBSSIの活性のない *wx* 変異体、*wx ae* 変異体のデンプンはともにアミロースを全く含んでおらずアミロペクチンのみからできていることが示された。*wx ae* 変異体のアミロペクチンの単位鎖長の長さは *wx* 変異体や元品種と比較して長くなっていた。この構造変化の結果、デンプン粒径の減少やB型結晶図形の変化などの構造や糊化温度の上昇や糊液の粘度上昇などの物性変化、アミラーゼによる消化性の低下などが引き起こされたと考えられた。

第3章では遺伝子の発現に及ぼす変化を High-Coverage Gene Expression Profiling 法を用いて網羅的に解析した結果について示している。データベース(KOME)に登録されている完全長 cDNA から分析可能と予測される遺伝子の総数は 21439 であり、この内、実験で 13046 の遺伝子の発現を検出することができた。この内、WTと比較して発現が2倍以上の遺伝子が 140 あり、発現量が 50%未満の遺伝子が 226 であった。糖質の代謝関連遺伝子に限ってみると、スクロースの代謝にかかわる *sucrose transporter1 (SUT1)* invertase a (*INVa*)と *sucrose sytase 3 (SuSy3)*の発現量が低下しており、主にデンプンの分解に関連する、*AAmy1A*、*AAmy3E*、*PhoH*、および *DPE2* は、上昇していた。二重変異体では、糖質代謝関連遺伝子以外にも発現に変動がみられた。特に脂質の合成にかかわる遺伝子の発現量増大やストレス抵抗性遺伝子発現の増大が観察された。二重変異体ではデンプンの合成量が減り、その結果スクロースの濃度が増大するが、代替の貯蔵化合物である脂質の生合成に使われているのではないかと考察された。また、高いスクロースの濃度は浸透圧ストレスを引き起こし、このことがストレス抵抗性に関連する遺伝子発現量の変化に影響を及ぼしたと推論している。

第4章では、変異によって遺伝子の発現が増大することが見いだされた *disproportionating enzyme 2* の遺伝子(*DPE2*)のクローニングと大腸菌での発現を行い酵素の性質を明らかにしている。この酵素がマルトースに特異的に働き、グルコース残基を水溶性グルカンに転移する働きがあることを示した。遺伝子発現の上昇が観察された *phosphorylase* の活性も上昇しており、これら二つの酵素の働きにより、二重変異体内のマルトースと可溶性グルカンの濃度が減少したと推測した。

本研究では、デンプンの合成にかかわる GBSSIと SBEIIb の酵素活性が失われた二重変異体を対象にして、そのデンプンの構造変化を明らかにするとともに、変異がどのように胚乳の遺伝子発現と代謝産物に影響を及ぼしているかについて多くの知見を得ている。特に、遺伝子の発現の増大が認められた *disproportionating enzyme 2* に注目して転移反応の詳細な実験を行い、マルトースと水溶性グルカン量が減少するメカニズムを解明した。また、二重変異体でスクロース濃度の増大が、脂質の合成関連遺伝子やストレス耐性遺伝子の発現量の増大に影響を及ぼしたとの示唆はこれからのイネの育種研究の端緒を見出した点で高く評価できる。よって、本論文の審査ならびに最終試験の結果と併せて、博士(応用生命科学)の学位を授与することを適当と認める。