Wxa遺伝子を発現した糯種イネ胚乳澱粉の分子構造

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Structures of Endosperm Starch from a Rice *wx* Cultivar Expressing *Wx*^{a} Transgene

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Abstract: Structural changes caused by the introduction of the *Wx*^{a} transgene, which encodes granule-bound starch synthase I (GBSSI), into the non-transgenic *wx* cultivar Musashimochi were examined and compared with the previous results (*Plant Cell Physiol.*, 49, 925–933 (2008), using a transgenic line (WAB3-5) established independently from those used previously. Increases of amylose content of starch and extra-long chain (ELC) content of amylopectin were observed in the line WAB3-5 (actual amylose, 15.2%; ELC, 11.6% by weight). An HPSEC profile (size distribution) of WAB3-5 amylose showed characteristics in-between of those of *Wx*^{a} and *Wx*^{b} cultivars, and WAB3-5 amylose had a comparably high degree of branching, which was indicated by the molar fraction of branched molecules (MF$_B$, 0.38) and number of chains of branched molecules ($\overline{NC}_B$, 10.5). Amylose from cv. Yumetoiro (*Wx*^{a}) had a comparable degree of branching (MF$_B$, 0.35; $\overline{NC}_B$, 13.7) with the WAB3-5 amylose. Chain-length distribution of WAB3-5 amylopectin showed a slightly higher amount of B_2+B_3 chains than cv. Musashimochi. These results are consistent with those of the previous study, providing additional evidence for the proposed role of GBSSI in both amylose and ELC synthesis in rice endosperm. ELCs of amylopectins from line WAB3-5 and cvs. IR36 and Yumetoiro showed size distributions that were basically similar to each other but distinct from those of their amylose counterpart. ELCs were hydrolyzed by β-amylolysis of amylopectins with different extents of trimming, 66.3, 92.0 and 77.0% for line WAB3-5, cv. IR 36 and cv. Yumetoiro, respectively, suggesting the branched structure of the ELCs is different among the three amylopectins.

Key words: *Wx*, granule-bound starch synthase, amylose content, extra-long chain

Amylose content of starch is a crucial factor determining various properties of starch and grains of rice (*Oryza sativa* L.). For example, correlation with amylose content and pasting properties of starch[1–3] or cooked rice textures[4,5] have been reported in the literature. Thus, as in other crops, attempts to manipulate amylose content have been common practice for generating rice starches with novel functionalities. Since rice has a relatively narrow range of amylose contents among major cereal crops, generating a real "high amylose" rice starch is expected to confer unique properties. In the previous studies, transgenic lines expressing the *Wx*^{a} (*WxR*) transgene, which encodes granule-bound starch synthase I (GBSSI), in endosperms of *wx* cultivars were generated,[6,7] and we examined the effects of the transgene on the structures and pasting properties of starch, showing that the expressed GBSSI synthesized amylose and extra-long chains (ELC) of amylopectin.[8] The aim of this study is to confirm the effects of the introduced GBSSI on structures of the starch components, using a transgenic line established independently from the lines used in the previous study[9] with a different vector and transformation method. Some selected starches were also examined for comparison: *Wx*^{b}-carrying cv. Nipponbare, *Wx*$^{a\text{-}Y224S}$-carrying cv. Labelle, and *Wx*^{a}-carrying cvs. IR36 and Yumetoiro, among which only cv. Labelle was used in the previous study. Further, besides ELC content, some structural characteristics, including difference in branched nature, of ELC synthesized in the transgenic line are compared with those of cvs. IR36 and Yumetoiro, both of which have been shown to have a significant amount of ELC.[10,11]

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MATERIALS AND METHODS

Materials. To establish transgenic lines, binary vector pWAB, which carries the *Wx*::*Wx*^{a} cDNA, was constructed.[6] Six kbp *Kpn*I/*Hin*dIII fragment of pWxR was ligated with *Kpn*I/*Hin*dIII digested pPZP2H-lac[12] and inserted into a multiple cloning site of the pPZP2H-lac. The resultant pWAB was introduced into *wx*-carrying cv. Musashimochi by *Agrobacterium*-mediated gene transfer.[9]

The copy numbers of the transgene per diploid of all R0 *WAB/wx* transgenic lines were determined by Southern blot analysis and then homozygous plants carrying a single copy of the *WAB* gene per haploid were selected for this study.[9] One of the transgenic lines, designated WAB3-5, showed normal fertility and the same phenotype as *WxR/wx* transgenic lines previously described.[6,7,9] The line WAB3-5 showed amylose accumulation at both pollen and endosperm[9] and was used for structural analysis of starch. For rice cvs. Nipponbare (carrying Wx^b), Musashimochi (*wx*), Labelle ($Wx^{a-Y224S}$), Yumetoiro (Wx^a) and IR36 (Wx^a), all seeds were sown in mid-April and cultivated in open fields. For the transgenic line WAB3-5, seeds were sown in mid-April, and the plants were cultivated in a closed greenhouse under natural light with occasional air-cooling for the temperature not to exceed 28–30°C during the daytime. Flowering dates varied from late-July to mid-August. Harvested seeds were used for starch purification. Cv. Yumetoiro was provided by Dr. N. Fujita (Akita Prefectural University, Akita, Japan) and cv. IR36 was provided by Prof. T. Tanisaka (Kyoto University, Kyoto, Japan). Starches were prepared from rice grains by alkaline steeping in cold. Amylose and amylopectin were fractionated from defatted starch by the method of Takeda *et al.*[13] ELC was obtained after butanol precipitation of debranched amylopectin.[14] *Pseudomonas* isoamylase and *Klebsiella* pullulanase were purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Sweet potato β-amylase (Sigma Chemical Co., USA) was purified by anion-exchange chromatography[15] prior to use. Other reagents were of the highest grade commercially available.

β-amylase limit dextrin (β-LD) of amylopectin. β-Amylolysis of amylopectin was performed as follows. Amylopectin (4 mg) was dissolved in H_2O (950 μL) by heating in a boiling-water bath and 1 M acetate buffer (pH 4.8, 50 μL) was added. The solution was transferred to a disposable dialysis cup (Daiichi Pure Chemicals Co., Ltd., Japan, molecular weight cut-off, 3500), and β-amylolysis (10 U/mg of amylopectin) was carried out at 37°C for 24 h with dialysis against 50 mM acetate buffer, pH 4.8. The reaction mixture was transferred to a glass test tube and the reaction was terminated by heating in boiling water for 5 min. The resulting β-amylase limit dextrin (β-LD) was precipitated by the addition of 3 volumes of ethanol, and after storage at −20°C for 2 h, the precipitate was collected by centrifugation at 2000 rpm for 10 min. The precipitate was washed 3 times by suspension in 3 mL of 75% ethanol containing 50 mM NaCl followed by centrifugation, and then the precipitate was dissolved in 1 mL of dimethyl sulfoxide in a boiling-water bath and after the addition of 3 mL of ethanol the mixture was kept at −20°C for 2 h. The precipitate was collected and washed twice as described above. β-LD was debranched by successive hydrolysis with isoamylase at 45°C for 12 h (0.2 U, pH 3.5) and pullulanase at 45°C for 6 h (0.2 U, pH 6.0). The hydrolyzate was concentrated to dryness with a centrifugal vacuum evaporator.

Analytical methods. Fluorescent labeling and subsequent size-exclusion HPLC (HPSEC) analysis of amylose[16] and debranched amylopectin[17] were performed according to the previous studies. β-Amylolysis of labeled amylose and subsequent HPSEC analysis for determination of the molar fraction of branched molecules (MFB) of amylose were followed as previously described.[18] The average number of chains (NC) of amylose was calculated based on determination of reducing and non-reducing residues.[19] For amylose, the average number of chains of branched molecules (NCB) was calculated by the following equation:[18] $NC_B=[NC-(1-MF_B)]/MF_B$.

RESULTS AND DISCUSSION

Amylose contents.

Apparent and actual amylose contents of starch were determined from chain-length distributions of starch and amylopectin.[8] Figure 1 shows chromatograms for the line WAB3-5 as example. Apparent content was obtained as a percentage of area of the peak eluted around the retention time of 60–70 min and actual content was obtained after subtraction of peak area corresponding to ELC of amylopectin. Table 1 summarizes *Wx* genotypes and amylose contents of starches of the rice cultivars and transgenic line used in this study. The designation $Wx^{a-Y224S}$ is used for cv. Labelle to indicate that the gene contains a single nucleotide substitution that results in replacement of tyrosine with serine residue at position 224 of the GBSSI protein.[8] The coding region of $Wx^{a-Y224S}$ is identical to that of Wx^{in}, which has been proposed as the putative allele that is responsible for intermediate amylose content.[20] Appar-

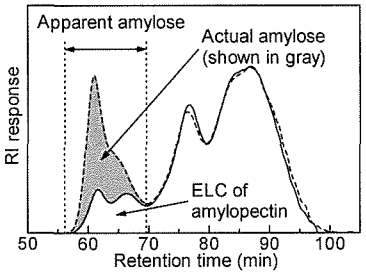

Fig. 1. Determination of apparent and actual amylose contents from chain-length distributions of starch and amylopectin.

Results for the line WAB3-5 is shown as an example. Solid line, amylopectin; dashed line, starch.

Table 1. Apparent and actual amylose contents of rice starches determined by HPSEC.

Variety	*Wx* genotype	Amylose content (% by weight)	
		Apparent	Actual
Musashimochi (M)	*wx*	—*	—
Labelle (L)	$Wx^{a-Y224S}$ (Wx^{in})**	20.6	18.7
WAB3-5	*WAB**** (Wx^a)	24.7	15.2
IR36	Wx^a	25.2	16.7
Yumetoiro (Y)	Wx^a	23.7	12.0
Nipponbare (N)	Wx^b	16.5	15.7

*Not detected. **Wx^a sequence with a nucleotide substitution that causes replacement of Y with S at position 224 of GBSSI protein.[8] Coding region of this gene is identical to that of Wx^{in}.[20] ***Wx::Wx^a cDNA fusion gene, which carries the 1st intron of Wx^a. The mRNA and protein of the *WAB* are identical to that of Wx^a.[6,7,9]

ent amylose content was increased by Wx^a (*WAB*) transgene expression from an undetectable amount (cv. Musashimochi) to 24.7% (line WAB3-5). The content in line WAB3-5 was comparable with that of cv. IR36 (Table 1), and slightly higher than those of the transgenic lines (21.6–22.2%) used in the previous study.[8] Apparent contents for cvs. Labelle,[21] IR36[10,22] and Nipponbare[23,24] were comparable with those reported in the literature except for ~6% lower content of cv. Yumetoiro.[11] Actual amylose content in line WAB3-5 was similar to that of cv. Nipponbare and the previous results (14.9–16.0%).[8] Thus, the effect on apparent and actual amylose contents of the Wx^a (*WAB*) transgene expression in line WAB3-5 was judged to be the same as in the transgenic lines used previously.

Structure of amylose.

Structures of amyloses are summarized in Table 2. Number-average degree of polymerization ($\overline{DP_n}$) was estimated by two methods, colorimetric and fluorescent labeling methods. The labeling method gave a higher $\overline{DP_n}$ than the colorimetric method for line WAB3-5, cvs. IR36 and Yumetoiro, and for the other cultivars $\overline{DP_n}$ by the two methods agreed well with each other. In comparisons of results by labeling method, WAB3-5 amylose had a similar $\overline{DP_n}$ value to those of cvs. Labelle and Nipponbare. The $\overline{DP_n}$ was slightly lower than the previously reported values for the other transgenic lines (1220–1240).[8] Cv. Labelle showed a $\overline{DP_n}$ that was rather closer to that of cv. Nipponbare than those of the Wx^a-carrying cultivars. Among the Wx^a-carrying cultivars, IR36 and Yumetoiro showed considerably higher $\overline{DP_n}$ than line WAB3-5. The number-average chain length ($\overline{CL_n}$) and average number of chains ($\overline{NC}$) of WAB3-5 amylose were the shortest and second highest, respectively, compared to other cultivars, indicating its comparably higher degree of branching. The molar fraction of branched molecules (MF_B) of WAB3-5 amylose was ~1.6-times higher than those of cvs. Labelle and IR36, and 1.4-times higher than that of cv. Nipponbare. The amylose of cv. Yumetoiro had a MF_B similar to that of line WAB3-5. The average number of chains of branched molecules ($\overline{NC_B}$) was highest for cvs. Labelle and Yumetoiro, followed by cv. IR36 and line WAB3-5. In comparison with cv. Nipponbare, the higher $\overline{MF_B}$ and $\overline{NC_B}$ of WAB3-5 amylose resulted in its shorter $\overline{CL_n}$ and higher $\overline{NC}$. Thus, WAB3-5 amylose can be characterized by the increased number of both branched molecules and branches (side chains) per branched molecule. Size distributions of amyloses examined by high-performance size-exclusion chromatography (HPSEC) are shown in Fig. 2. These distributions can be arranged in the order of N, L, WAB3-5, IR36 and Y, based on appearance of two shoulders, one in a fluorescent profile around the retention time of 80 min and the other in an RI profile around the retention time of 85 min. The appearance of these shoulders changes in opposite ways, increasing and decreasing for fluorescent and RI profiles, respectively, in the order mentioned above, and the changes are in parallel to each other. Thus, the size distribution of WAB3-5 amylose exhibited characteristics in-between those of Wx^a and Wx^b cultivars. The characteristics of WAB3-5 amylose in size ($\overline{DP_n}$ and its distribution) and branching degrees (MF_B and $\overline{NC_B}$) are consistent with the previous observation for other transgenic lines expressing the Wx^a (*WxR*) transgene.[8]

Table 2. Structures of rice amyloses.*

Variety	$\overline{DP_n}$		$\overline{CL_n}$	$\overline{NC}$	MF_B	$\overline{NC_B}$
	Colorimetric	Labeling				
L	1100	1190	270	4.1	0.23	14.5
WAB3-5	870	1120	190	4.6	0.38	10.5
IR36	1090	1400	330	3.3	0.24	10.6
Y	1140	1480	210	5.4	0.35	13.7
N	1090	1160	350	3.1	0.27	8.8

*Abbreviations: $\overline{DP_n}$, number-average degree of polymerization; $\overline{CL_n}$, number-average chain length; $\overline{NC}$, average number of chains, calculated as $\overline{DP_n}$ (colorimetric)/$\overline{CL_n}$); MF_B, molar fraction of branched molecules; $\overline{NC_B}$, average number of chains of branched molecules, calculated as $[\overline{NC}-(1-MF_B)]/MF_B$.

Structure of amylopectin.

Chain-length (CL) distributions of amylopectins (Fig. 3) showed that addition of ELCs is the most prominent ef-

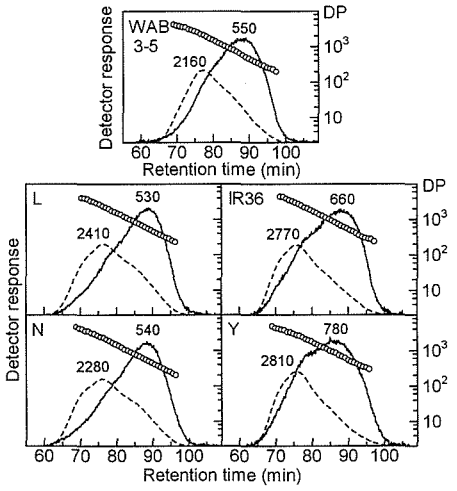

Fig. 2. Size distributions of amyloses.

Solid line, fluorescence; dashed line, RI; open circle, DP at a given elution position. Numbers indicate peak-top DP.

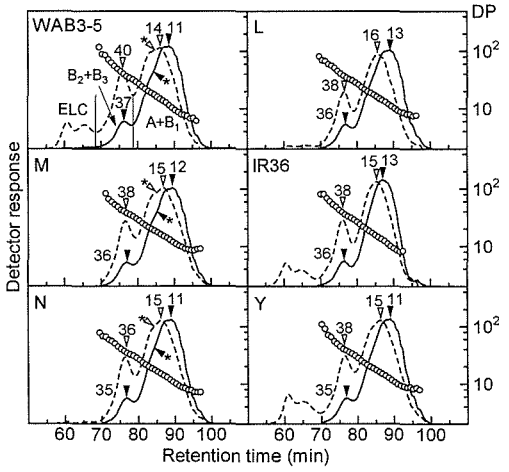

Fig. 3. Chain-length distributions of amylopectins.

Symbols are as shown in Fig. 2. Arrowheads with asterisks indicate shoulders around DP 18.

Table 3. Chain-length distributions of amylopectins.

Variety	Amount (% by mol)			A+B$_1$*	Amount (% by weight)			$\overline{CL_n}$	
	ELC	B$_2$+B$_3$	A+B$_1$	B$_2$+B$_3$	ELC	B$_2$+B$_3$	A+B$_1$	B$_2$+B$_3$	A+B$_1$
M	—**	8.9	91.1	10.2	—	23.3	76.7	43	14
L	0.3	9.8	89.9	9.2	2.3	24.2	73.5	41	14
WAB3-5	0.8	10.0	89.2	8.9	11.6	24.9	63.5	44	14
IR36	0.5	9.7	89.8	9.3	10.2	22.6	68.4	44	14
Y	0.6	9.0	90.4	10.0	12.4	21.6	66.0	45	14
N	0.2	9.1	90.7	10.0	0.9	23.7	75.4	41	13

*Molar ratio of unit-chain fractions. **Not detected.

fect of the transgene expression (see panels WAB3-5 and M). Fractions were designated as shown in the figure and their amounts were compared (Table 3). On a molar basis, WAB3-5 amylopectin contained decreased and increased amounts of A + B$_1$ and B$_2$ + B$_3$ fractions, respectively, in comparison to cv. Musashimochi. The changes were more clearly seen on a weight basis even though a significant amount of ELC fraction is included. The molar ratio of the fractions (A + B$_1$)/(B$_2$ + B$_3$) was altered accordingly. The changes in these two fractions were observed only in the amounts but not in $\overline{CL_n}$. The shoulder around DP18 that was seen in cv. Musashimochi was unchanged and similarly observed in line WAB3-5. The ELC addition and other changes found in WAB3-5 amylopectin agreed well with the previous results.[8] In comparison with other cultivars, WAB3-5 amylopectin had in common with those of cvs. Musashimochi and Nipponbare the presence of a clear shoulder around DP18 (Fig. 3), which was seen more easily in RI than in the fluorescence profile. However, the molar and weight amount of B$_2$ + B$_3$ fraction and the molar ratio of (A + B$_1$)/(B$_2$ + B$_3$) were closer to cvs. Labelle and IR36, and $\overline{CL_n}$ of B$_2$ + B$_3$ fraction was comparable with those of cvs. IR36 and Yumetoiro (Table 3).

Structure of extra-long chains (ELCs) of amylopectin.

ELCs were fractionated by butanol precipitation of debranched amylopectin[14] from line WAB3-5 and cvs. IR36 and Yumetoiro, and then analyzed with HPSEC (Fig. 4).[16] Values of peak-top DP of fluorescent profiles were similar but those of RI profiles slightly differed among the three specimens, and their order was the same as that of $\overline{DP_n}$. These values of $\overline{DP_n}$ and peak-top DP as well as elution profiles are also comparable with those reported previously for rice cvs. IR36 and IR42.[14] To probe how these ELCs are connected to amylopectin molecules, β-amylase limit dextrin (β-LD) was prepared and trimming of ELCs by the enzyme was estimated by comparison of CL distributions of amylopectin and its limit dextrin (Fig. 5). In cvs. Labelle and Nipponbare, the ELC contents in amylopectin were very low and the ELCs were not detectable after β-amylolysis (data not shown). The extent of trimming of ELC by the enzyme was different among line WAB3-5 and cvs. IR36 and Yumetoiro (66.3, 92.0 and 77.0%, respectively, estimated from the decrease in the peak area of the ELC fraction). Two peaks were seen in the ELC fraction in each amylopectin, and they appeared to be differentially hydrolyzed. The first peak seemed to

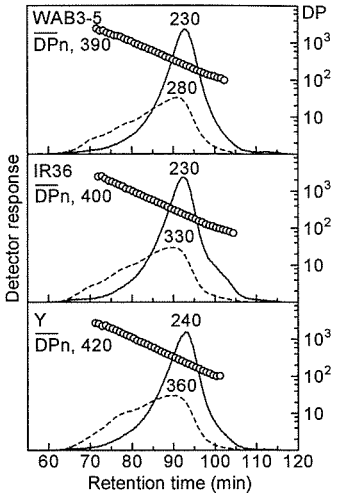

Fig. 4. Size distributions of ELCs.

Symbols are as shown in Fig. 2.

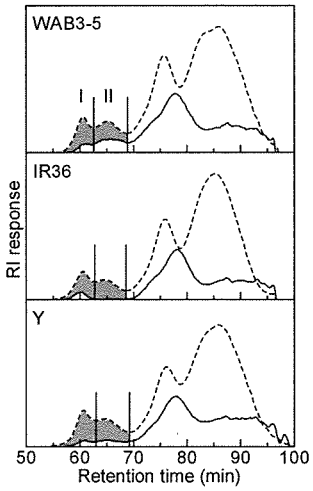

Fig. 5. Chain-length distributions of amylopectins and their β-LDs.

Solid line, β-LD; dashed line, amylopectin. The two peaks in the ELC fraction are labeled as I and II in the order of elution.

be degraded to a rather similar extent for the three amylopectins while the extent of trimming of the second peak, which accounts for *ca.* 80–90% of the ELC fraction on a molar basis,[14] differed greatly among the specimens. Since hydrolysis by β-amylase, an exo-type enzyme, is hindered by the presence of side chains,[25] the difference can be explained by the number of side chains attached to ELC and/or the position (how far from a non-reducing end) of side chains on ELC. Although it could not be exactly elu-

cidated which of the two was the major cause for the observed difference, at least it was shown that the ELCs examined from the three amylopectins were different in those aspects, namely, branching structure of ELCs.

In summary, structural changes observed in this study in the rice starch of transgenic line WAB3-5, which were due to Wx^a (*WAB*) transgene expression in a *wx* cultivar, were consistent with those of the previous study[8] in aspects such as: amylose content; structures of amylose; ELC addition to amylopectin; and increased amount of $B_2 + B_3$ chains of amylopectin. Thus the confirmation using the transgenic line strongly supported the role of GBSSI in starch biosynthesis in rice endosperm proposed previously,[8] that is, synthesis of both amylose and ELC of amylopectin. This study also suggested that ELC of WAB 3-5 amylopectin had a different branched structure from those of cvs. IR36 and Yumetoiro, which also showed difference from each other.

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Wx^a 遺伝子を発現した糯種イネ胚乳澱粉の分子構造

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粒結合型澱粉合成酵素 I (GBSSI) をコードする *Wx*^{a} 遺伝子を糯種イネ (ムサシモチ) に導入し，澱粉の構造へ与える影響を調べ，以前の結果 (*Plant Cell Physiol.*, **49**, 925-933 (2008)) と比較した．トランスジェニック系統 WAB3-5 では，澱粉のアミロース含量 (15.2%) とアミロペクチンの超長鎖含量 (ELC, 11.6%) が増加していた (Table 1)．WAB3-5 のアミロースは，重合度分布については *Wx*^{a} 型の品種と *Wx*^{b} 型の日本晴との中間的な特徴を有し (Fig. 2)，分岐分子のモル分率 (MF_B, 0.38) および分岐分子の平均鎖数 ($\overline{NC}_B$, 10.5) で示される分岐の程度は非常に高かった (Table 2)．夢十色アミロースの分岐の程度はこれに近かった (MF_B, 0.35; $\overline{NC}_B$, 13.7)．アミロペクチンの鎖長分布では $B_2 + B_3$ 鎖の量が WAB3-5 でわずかに増加していた (Table 3)．これらの構造変化は以前の結果とよく一致しており，既報で提唱したイネ胚乳でのアミロースと ELC の合成における GBSSI の役割を支持した．また，WAB3-5，IR36，夢十色の ELC を比較したところ，これらの重合度分布は基本的には類似しており (Fig. 4)，それぞれのアミロースとは異なっていた．アミロペクチンに β-アミラーゼを作用させた際の ELC の分解率は大きく異なったことから (WAB3-5, 66.3; IR36, 92.0; 夢十色, 77.0%)，各々異なる分岐構造を持つことが示唆された (Fig. 5).