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Verification of the Bayer CropScience 35S-BAR Method for the Detection of LL62/LL601 Rice Using Real-Time PCR

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Executive Summary

The Technical Services Division of USDA's Grain Inspection, Packers and Stockyards Administration conducted a study to verify the performance of a construct-specific method to detect the Liberty Link events, LL62 and LL601 in rice. Bayer CropScience provided the 100% LL62 and LL601 rice in the form of whole-grain, rough rice as well as the written protocol for performing the PCR reactions.

Results from this study confirm that the 35S-BAR method can successfully detect both the LL601 and LL62 events in long-grain rice samples at a level as low as 0.01% for either event.

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1. Background

The purpose of this study is to provide independent verification of the performance of the Bayer CropScience method for the construct-specific detection of the genetically-engineered rice varieties, LL601 and LL62. A key component of this verification includes determination of the limit of detection in LL62 and LL601 in fortified samples using 35S-BAR primers and probe. The protocol is a real-time TaqMan® PCR procedure for determination of the relative content of LL62/LL601 DNA to total rice species DNA in a sample. The construct-specific protocol uses two specific primers for 35S-BAR and a construct-specific oligonucleotide FAM/MGB probe that amplifies to an unknown bp fragment. A PCR amplified product is measured during each cycle of the reaction process. Relative quantification of an endogenous control is achieved by amplification of a rice-specific 68-bp fragment of *Phospholipase D* (PLD), also described as "*Oryza sativa*" by Bayer CropScience. This rice-specific endogenous gene employs gene-specific primers and a sequence-specific FAM/TAM probe. The PCR reactions for the target and reference genes are performed in separate wells (simplex).

Bayer Crop Science provided GIPSA with long-grain (rough) rice containing 100% LL601, 100% LL62, samples fortified with low levels of LL62/LL601, and the construct-specific protocol for detection of the 35S-BAR. Certified reference material for LL62 was obtained through the American Oil Chemist Society (AOCS) in the form of rice genomic DNA at 10 µg/vial (catalog number 0306-I). GIPSA non-Transgenic rice 13/16 (GNTR13/16) DNA samples, derived from long-grain rice, was obtained from GIPSA file samples and used as the control blank.

2. Experimental Plan

Initial work using the "Dellaporta-derived Method for DNA Extraction..." provided by Bayer Crop Science did not provide sufficient quantities of rice genomic DNA for efficient PCR amplification. Therefore, a publicly available CTAB extraction method was used but required an additional RNAase A (Fermentas, catalog number EN0531) digestion step at the end of the extraction procedure to remove excessive amounts of contaminating RNA.

Sensitivity studies were performed on long-grain rice samples serially diluted into GNTR13/16 at 1%, 0.1%, 0.05%, 0.01% and 0.005% LL62 or LL601 or non-fortified GNTR13/16. Each sample was amplified as a simplex format using: (1) 35S-BAR construct-specific primers/probe and (2) endogenous control PLD primers/probe. Sensitivity studies were performed on DNA extracts of LL62 and LL601 diluted with GNTR13/16 DNA to maintain a constant rice DNA level of 200 ng per reaction. In addition, two separate gravimetrically fortified samples containing: (1) 1% LL62 and (2) 1% LL601 were extracted using the CTAB protocol as described above and used in this study. All PCR reactions were run in triplicate except reference standards were run in duplicate.

3. Experimental Results

3.1 DNA Extraction and Quantification

Rice genomic DNA was extracted using minor modifications of a publicly available CTAB extraction protocol. A second RNAase A incubation at 65°C for 30 minutes was required upon isolation of rice genomic DNA, followed by phenol/chloroform extraction and ethanol precipitation. The DNA was

quantified using a pico-green reagent kit from Molecular Probes (catalog # P7589). Upon quantification of individual samples, each concentration was adjusted to 40 ng/µl using 0.5 x TE (Tris-EDTA, pH 8.0) buffer. The integrity of the isolated rice DNA was characterized using agarose gel electrophoresis by size separation on a 0.8% agarose gel set at 100 volts for 30 minutes. A total of 200 ng DNA (5µl) was loaded into individual wells and onto the gel. Intact, high molecular weight rice genomic DNA was observed in all samples but frequently contained some RNA contamination. The low levels of RNA contamination did not seem to affect the PCR efficiency.

3.2 PCR Reaction Protocol

Table 1. Reagents and concentrations per reaction well for the 35S-BAR system

Reagent	Concentration Stock	Final Concentration	µl per 1 rxn
H_2O	-	-	5
MDB498	10 µM	400nM/rxn	1
DPA143	10 µM	400nM/rxn	1
TM099	10 µM	200nM/rxn	0.5
Taq polymerase, ABI master mix	2x concentrate	1x concentrate	12.5
DNA	40 ng/ µl	200 ng/rxn	5
Total Volume			25

Table 2. Reagents and concentrations per reaction well for the PLD reference system

Reagent	Concentration Stock	Final Concentration	µl per 1 rxn
H_2O	-	-	6
KVM159	10 µM	200nM/rxn	0.5
KVM160	10 µM	200nM/rxn	0.5
TM013	10 µM	200nM/rxn	0.5
Taq polymerase, ABI master mix	2x concentrate	1x concentrate	12.5
DNA	40 ng/ µl	200 ng/rxn	5
Total Volume			25

The PCR reactions were performed with ABI universal PCR master mix reagent (catalog number 4304437), a commercially available Taq DNA polymerase enzyme purchased from Applied Biosystems, Inc. Ten µg of rice Certified Reference Material (100% LL62) was purchased from the American Oil Chemists Society (AOCS, catalog number 0306-I). Primers and probes for both the reference specific PLD and target specific (LL601) genes are shown below:

35S-BAR Target Reaction		
Name	*Description*	*5'– 3' sequence*
MDB498	Forward Primer	TAT CCT TCG CAA GAC CCT TCC
DPA143	Reverse Primer	ATG TCG GCC GGG CGT CGT TCT G
TM099	Probe	6FAM-TCT ATA TAA GGA AGT TCA TTT CAT T-MGBNFQ

Phospholipase D Reference System Reaction		
Name	*Description*	*5'– 3' sequence*
KVM159	Forward Primer	TGG TGA GCG TTT TGC AGT CT
KVM160	Reverse Primer	CTG ATC CAC TAG CAG GAG GTC C
TM013	Probe	FAM-TGT TGT GCT GCC AAT GTG GCC TG-TAMRA

Table 3. Two-step PCR amplification reaction conditions

Stage	Temp	Time	Cycles
UNG	50 °C	2 min	1
Initial denaturation	95 °C	10 min	1
Amplification	95 °C	15 sec	45
	60 °C	1 min	

Negative control reactions:

(1) Reagent blank sample that went through the entire purification procedure
(2) GNTR13/16- A GIPSA derived file sample of long-grain rice (repeated testing demonstrated to be negative)

Positive control reactions:

(1) Ground 100% Pure LL62- gravimetrically fortified at 1% (w/w%) into GNTR13/16 ground rice. DNA was isolated using the CTAB protocol as described.
(2) Ground 100% Pure LL601- gravimetrically fortified at 1% (w/w%) into GNTR13/16 ground rice. DNA was isolated using the CTAB protocol as described.
(3) 10ng, 1ng, 0.1ng, 0.01ng rice genomic DNA derived from 100% LL601 (40 ng/µl) serially diluted into 0.5x TE buffer and amplified with primers/probes specific for 35S-BAR. The 35S-BAR was amplified using PCR reaction conditions as described in this report.
(4) 200ng, 100ng, 10ng, 1ng, 0.1ng, 0.01 ng rice genomic DNA derived from 100% GNTR13/16 (40ng/µl) serially diluted into 0.5x TE buffer and amplified with primers/probes specific for PLD. The endogenous control gene was amplified using PCR reaction conditions as described in this report.

(5) 200ng, 100ng, 10ng, 1ng, 0.1ng, 0.01ng rice genomic DNA derived from AOCS 100% LL62 (40ng/µl) serially diluted into 0.5xTE buffer and amplified with primers/probe specific for the 35S-BAR. The 35S-BAR was amplified using PCR reaction conditions as described in this report.
(6) 200ng, 100ng, 10ng, 1ng, 0.1ng, 0.01ng rice genomic DNA derived from AOCS 100% LL62 (40ng/µl) serially diluted into 0.5x TE buffer and amplified with primers/probes specific for PLD. The endogenous control gene was amplified using PCR reaction conditions as described in this report.

Test Samples:
DNA was extracted and quantified for rice samples containing 100% LL601, 1% LL601 (prepared by GIPSA), 1% LL62 (prepared by GIPSA) using the CTAB extraction and isolation procedures given in this report.

Sensitivity testing was performed by diluting the 100% LL62 (AOCS) or 100% LL601 (GIPSA) DNA with non-transgenic GNTR13/16, long-grain rice blank DNA, at the levels of 1%, 0.1%, 0.05%, 0.01%, and 0.005%. The total rice DNA content was maintained at 200 ng for these experiments. All PCR reactions amplified consistently for both the reference samples and target down to 0.01%. At 0.005%, three of three showed successful amplification for the 35S-BAR detection of LL601 and three of three showed successful amplification for the 35S-BAR detection of LL62 at 0.01%. None of the GNTR13/16 blank samples amplified for the 35S-BAR but did amplify for the endogenous control.

4. Conclusions

Based on the experiments conducted, the Bayer CropScience real-time construct-specific method for 35S-BAR in rice showed a limit of detection of 0.01% for LL62 and 0.01% for LL601 in long-grain rice samples using the CTAB extraction protocol and 200 ng of rice genomic DNA in the PCR reaction. The data presented in this report confirms that the 35S-BAR method is applicable for the detection of LL62 and LL601 in long-grain rice and the method can be used as a semi-quantitative or qualitative detection method. The method does not discern between the two LL traits. The results of this study suggest that a test sample, containing neither LL601 nor LL62 at or above the limit of detection for the method, will provide a negative result for 35S-BAR. Thus, the 35S-BAR method can be used as a screening tool and potentially eliminates the need to perform event-specific testing by laboratories.

Table 4. Sensitivity results for LL601 using the 35SBAR method

Well	Sample Name	Detector Name	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty
A1	GNTR13/16 200	TM013	Standard	20.31	0.092	200		
A2	GNTR13/16 200	TM013	Standard	20.44	0.092	200		
A3	GNTR13/16 100	TM013	Standard	21.23	0.019	100		
A4	GNTR13/16 100	TM013	Standard	21.21	0.019	100		
A5	GNTR13/16 10	TM013	Standard	24.23	0.025	10		
A6	GNTR13/16 10	TM013	Standard	24.2	0.025	10		
A7	GNTR13/16 1	TM013	Standard	27.34	0.034	1		
A8	GNTR13/16 1	TM013	Standard	27.39	0.034	1		
A9	GNTR13/16 0.1	TM013	Standard	31.08	0.118	1.00E-01		
A10	GNTR13/16 0.1	TM013	Standard	30.91	0.118	1.00E-01		
A11	GNTR13/16 0.01	TM013	Standard	34.05	0.267	1.00E-02		
A12	GNTR13/16 0.01	TM013	Standard	34.43	0.267	1.00E-02		
B1	1% 601 grav	TM013	Unknown	22.67	1.251	34.26	34.28	7.75E-01
B2	1% 601 grav	TM013	Unknown	22.7	1.251	33.52	34.28	7.75E-01
B3	1% 601 grav	TM013	Unknown	22.64	1.251	35.07	34.28	7.75E-01
B4	1% 601 ser	TM013	Unknown	20.36	1.251	177.66	173.95	6.0127
B5	1% 601 ser	TM013	Unknown	20.36	1.251	177.15	173.95	6.0127
B6	1% 601 ser	TM013	Unknown	20.45	1.251	167	173.95	6.0127
B7	0.1% 601	TM013	Unknown	20.39	0.003	173.62	173.34	3.45E-01
B8	0.1% 601	TM013	Unknown	20.39	0.003	173.45	173.34	3.45E-01
B9	0.1% 601	TM013	Unknown	20.4	0.003	172.95	173.34	3.45E-01
B10	0.01% 601	TM013	Unknown	20.37	0.126	176.37	170.04	14.874
B11	0.01% 601	TM013	Unknown	20.57	0.126	153.04	170.04	14.874
B12	0.01% 601	TM013	Unknown	20.33	0.126	180.7	170.04	14.874
C1	0.005 601	TM013	Unknown	20.41	0.046	171.67	176.06	5.757
C2	0.005 601	TM013	Unknown	20.32	0.046	182.57	176.06	5.757
C3	0.005 601	TM013	Unknown	20.39	0.046	173.93	176.06	5.757
C4	GNTR13/16 blank	TM013	Unknown	20.36	0.047	177.45	183.48	6.091
C5	GNTR13/16 blank	TM013	Unknown	20.31	0.047	183.35	183.48	6.091
C6	GNTR13/16 blank	TM013	Unknown	20.27	0.047	189.63	183.48	6.091

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
E1	601 10 ng	TM099	FAM/MGB	Standard	26.531301	10		
E2	601 10 ng	TM099	FAM/MGB	Standard	26.392748	10		
E3	601 1ng	TM099	FAM/MGB	Standard	29.694788	1		
E4	601 1ng	TM099	FAM/MGB	Standard	29.828922	1		
E5	601 0.1ng	TM099	FAM/MGB	Standard	32.20716	0.1		
E6	601 0.1ng	TM099	FAM/MGB	Standard	32.325153	0.1		
E7	601 0.01 ng	TM099	FAM/MGB	Standard	35.682335	0.01		
E8	601 0.01 ng	TM099	FAM/MGB	Standard	34.93274	0.01		
F1	1% 601 ser	TM099	FAM/MGB	Unknown	29.197227	1.26866	1.2148265	0.07969967
F2	1% 601 ser	TM099	FAM/MGB	Unknown	29.213345	1.2525511	1.2148265	0.07969967
F3	1% 601 ser	TM099	FAM/MGB	Unknown	29.350742	1.1232684	1.2148265	0.07969967
F4	1% 601 grav	TM099	FAM/MGB	Unknown	30.248735	0.5511454	0.6708072	0.12285321
F5	1% 601 grav	TM099	FAM/MGB	Unknown	30.012545	0.6646557	0.6708072	0.12285321
F6	1% 601 grav	TM099	FAM/MGB	Unknown	29.784124	0.79662067	0.6708072	0.12285321
F7	0.1% 601	TM099	FAM/MGB	Unknown	32.906006	0.067027725	0.06765888	0.002725096
F8	0.1% 601	TM099	FAM/MGB	Unknown	32.83973	0.07064418	0.06765888	0.002725096
F9	0.1% 601	TM099	FAM/MGB	Unknown	32.93885	0.06530475	0.06765888	0.002725096
F10	0.05% 601	TM099	FAM/MGB	Unknown	34.089867	0.026218068	0.032687332	0.005611415
F11	0.05% 601	TM099	FAM/MGB	Unknown	33.681683	0.03623731	0.032687332	0.005611415
F12	0.05% 601	TM099	FAM/MGB	Unknown	33.703827	0.03560662	0.032687332	0.005611415
G1	0.01% 601	TM099	FAM/MGB	Unknown	36.011154	0.00571504	0.004638392	0.001846477
G2	0.01% 601	TM099	FAM/MGB	Unknown	36.015842	0.005693836	0.004638392	0.001846477
G3	0.01% 601	TM099	FAM/MGB	Unknown	37.050777	0.002506299	0.004638392	0.001846477
G4	0.005 601	TM099	FAM/MGB	Unknown	36.90814	0.002806399	0.002803894	1.96E-04
G5	0.005 601	TM099	FAM/MGB	Unknown	37.001293	0.002606588	0.002803894	1.96E-04
G6	0.005 601	TM099	FAM/MGB	Unknown	36.82455	0.002998695	0.002803894	1.96E-04
G7	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
G8	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
G9	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
Slope	-2.9040837	cycles/log decade						
Y-Intercept		29.49735						
R^2		0.9939015						

Table 5. Sensitivity results for LL62 using the 35SBAR method

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
1	200ng	TM013	FAM	Standard	19.758347	200		
2	200ng	TM013	FAM	Standard	19.556015	200		
3	100ng	TM013	FAM	Standard	21.03312	100		
4	100ng	TM013	FAM	Standard	21.008373	100		
5	10ng	TM013	FAM	Standard	24.380268	10		
6	10ng	TM013	FAM	Standard	24.342522	10		
7	1.0ng	TM013	FAM	Standard	27.78911	1		
8	1.0ng	TM013	FAM	Standard	27.80842	1		
9	0.1ng	TM013	FAM	Standard	31.152197	0.1		
10	0.1ng	TM013	FAM	Standard	31.015432	0.1		
11	0.01ng	TM013	FAM	Standard	34.619286	0.01		
12	0.01ng	TM013	FAM	Standard	34.96806	0.01		
13	1% 62 grav	TM013	FAM	Unknown	20.399412	135.40671	143.35945	7.226308
14	1% 62 grav	TM013	FAM	Unknown	20.25028	149.52328	143.35945	7.226308
15	1% 62 grav	TM013	FAM	Unknown	20.294937	145.14833	143.35945	7.226308
19	0.1% 62	TM013	FAM	Unknown	20.813375	102.82276	112.811005	8.741028
20	0.1% 62	TM013	FAM	Unknown	20.59285	119.062805	112.811005	8.741028
21	0.1% 62	TM013	FAM	Unknown	20.62496	116.54746	112.811005	8.741028
25	0.01% 62	TM013	FAM	Unknown	20.77538	105.4538	106.41043	3.2545543
26	0.01% 62	TM013	FAM	Unknown	20.711414	110.03609	106.41043	3.2545543
27	0.01% 62	TM013	FAM	Unknown	20.8	103.7414	106.41043	3.2545543
31	0% GNTR13/16	TM013	FAM	Unknown	20.624512	116.58221	122.75875	6.082268
32	0% GNTR13/16	TM013	FAM	Unknown	20.475311	128.74214	122.75875	6.082268
33	0% GNTR13/16	TM013	FAM	Unknown	20.544514	122.951904	122.75875	6.082268
Slope	-3.4626522	cycles/log decade						
Y-Intercept		27.78054						
R^2		0.99923146						

Well	Sample Name	Detector Name	Reporter	Task	Ct	Quantity	Qty Mean	Qty StdDev
49	200	TM099	FAM/MGB	Standard	17.813646	200		
50	200	TM099	FAM/MGB	Standard	17.658653	200		
51	100	TM099	FAM/MGB	Standard	19.135065	100		
52	100	TM099	FAM/MGB	Standard	19.285973	100		
53	10	TM099	FAM/MGB	Standard	22.24516	10		
54	10	TM099	FAM/MGB	Standard	22.424803	10		
55	1	TM099	FAM/MGB	Standard	25.944822	1		
56	1	TM099	FAM/MGB	Standard	25.863056	1		
57	0.1	TM099	FAM/MGB	Standard	29.275665	0.1		
58	0.1	TM099	FAM/MGB	Standard	29.290178	0.1		
59	0.01	TM099	FAM/MGB	Standard	32.51954	0.01		
60	0.01	TM099	FAM/MGB	Standard	32.803844	0.01		
61	1% 62 grav	TM099	FAM/MGB	Unknown	24.863552	1.9226526	2.1088436	0.1617991
62	1% 62 grav	TM099	FAM/MGB	Unknown	24.670464	2.188572	2.1088436	0.1617991
63	1% 62 grav	TM099	FAM/MGB	Unknown	24.652367	2.2153058	2.1088436	0.1617991
67	0.1% 62	TM099	FAM/MGB	Unknown	29.38893	0.09233082	0.08479381	0.006688679
68	0.1% 62	TM099	FAM/MGB	Unknown	29.556986	0.08248601	0.08479381	0.006688679
69	0.1% 62	TM099	FAM/MGB	Unknown	29.610733	0.07956462	0.08479381	0.006688679
73	0.01% 62	TM099	FAM/MGB	Unknown	32.394478	0.012292026	0.011783649	0.00139996
74	0.01% 62	TM099	FAM/MGB	Unknown	32.672478	0.010200533	0.011783649	0.00139996
75	0.01% 62	TM099	FAM/MGB	Unknown	32.327335	0.012858392	0.011783649	0.00139996
79	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
80	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
81	0% GNTR13/16	TM099	FAM/MGB	Unknown	Undetermined	0		
Slope	-3.4320755	cycles/log decade						
Y-Intercept		25.837921						
R^2		0.99911314						