Development of the In-House Genetically Modified Wheat MON71800 Reference Plasmid for Qualitative Detection by Tetraplex Real-Time PCR.

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Abstract

Importation of GM plants to Thailand is prohibited except GM corn and soybean only for the industry supply propose. In 2019, Thailand imported more than 3 million tons of wheat, which MON71800 is the only GM wheat that appears in the ISAAA approval database. A screening test for identifying GM wheat contamination in imported wheat to Thailand is necessary. CaMV35S promoter and NOS terminator are the main elements mostly found in GM plants. To distinguish GM wheat from other GM plants, event-specific detection of MON71800 is needed to be employed with ACC-1, wheat endogenous control. Tetraplex Real-Time PCR is a technique that can detect the CaMV35S promoter, NOS terminator, event-specific MON71800, and ACC-1 in one reaction. In GM wheat MON71800 screening service, certified reference material (CRM) of MON71800 is essential for use as a positive control. Unfortunately, MON71800 CRM has not been commercialized. Therefore, the authors have developed an in-house plasmid that contains the elements of the CaMV35S promoter, NOS terminator, event-specific MON71800, and ACC-1. Moreover, the authors reveal specific primer and probe sequence and detection conditions by Tetraplex Real-Time PCR. This study is useful for screening tests of GM wheat and economizes by reducing the cost of CRM and chemical use in Real-Time PCR reaction.

Keywords**:** *MON71800, Tetraplex Real-Time PCR, reference plasmid, CaMV35S promoter, NOS terminator, ACC-1*

1. Introduction

Modern biotechnology is a powerful tool and useful for agriculture by improving and reducing the time of plant breeding program to produce a plant that contains specific and functional traits such as drought tolerance, salinity tolerance, herbicide tolerance, disease resistance, and increase nutrition. Over the past 25 years, the event of genetically modified (GM) has greatly increased (1996-2019). Moreover, the global area of GM crop cultivation has increased more than 100 folds (1.7 million hectares in 1996 to 189.8 million hectares in 2017), and the top 5 global GM crop planted areas were soybean, corn, cotton, canola, and alfalfa (ISAAA, 2017).

One of the major staple food crops which grew over 17% of global cultivation land is wheat (*Triticum aestivum* L.) (Xia et al., 2012). The development of GM wheat trait by biotechnology for herbicide tolerance and disease resistance have been improved for decades (Leckband & Lörz 1998; Anand et al. 2003; Hu et al. 2003; Abouseadaa et al. 2015; Shin et al. 2008). MON71800, Glyphosate tolerance wheat developed by Monsanto, has been launched in the 90s and authorized for test field trials in the USA. After finishing the program, there was no presence of MON71800 in the commercial market. However, unexpected MON71800 was discovered, noticed, and confirmed in Oregon by the United States Department of Agriculture (USDA) in 2013.

Most of the consuming wheat in Thailand is imported from foreign countries. In 2019, Thailand imported 3,630,526,173 kg of wheat for supply in the food and feed industry (The office of Agriculture Regulation, Department of Agriculture, Thailand). According to Thailand's plant quarantine act, B.E. 2507 (1952) amended (No.2) B.E. 2542 (1999) and (No.3) B.E. 2551 (2008), importation of GM plant is prohibited, exempting the importation of soybean and corn for industry supply and other species of plants for research, in compliance with the notification of Department of Agriculture's Guideline. To concern food safety and to follow Thailand's plant quarantine act, the importation of wheat to Thailand needs to be examined for screening detection of GM wheat contamination.

Several studies of the European Commission Joint Research Center (EU-JRC) have been reported the test method for detection of MON71800 by specific primer and probe by Real-Time PCR in EU-JRC

webpage (https://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). Since reference material of MON71800 as a positive control has not been commercialized, an in-house plasmid control for MON71800 was developed and designed to contain event-specific sequences of MON71800 and wheat endogenous gene (Kim et al., 2015).

Most GM food and feed contain CaMV35S promoter and NOS terminator in their genome. A PCR-based method is a common technique for qualitative screening tools of GM food and feed worldwide. Real-Time PCR is one of the standard techniques mostly applied in ISO/IEC 17025:2017 laboratory for screening tests of GM food and feed service, which is sensitive and needs high precision. However, in comparison with a traditional PCR, the cost of chemical material and instruments is higher (Randhawa et al., 2016). Multiplex Real-Time PCR is an economical technique that can be applied for screening many genes in one reaction (Huber et al., 2013). Previously, the authors revealed Triplex Real-Time PCR of CaMV35S promoter, NOS terminator, and HMG, corn endogenous control gene, for the screening of GM corn in ISO/IEC17025 standard accreditation laboratory (Thammakijjawat et al., 2018). In this study, the authors produced the in-house positive reference plasmid of GM wheat MON71800 that contains the CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 wheat endogenous control to use as a positive control in the Tetraplex Real-Time PCR.

2. Objectives

1) To develop an in-house positive reference plasmid of GM wheat MON71800.

2) To evaluate the feasibility of using the in-house positive reference plasmid of GM wheat MON71800 in Tetraplex Real-Time PCR for a screening of CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 wheat endogenous control in one reaction by Tetraplex Real-Time PCR.

3. Materials and Methods

3.1 Positive reference plasmid design

The reference plasmid was constructed and cloned into a pUC57 vector, and the positive reference plasmid was named pUC57-MON71800. The sequence of insertion fragment consists of the CaMV35S promoter, MON71800 event-specific element, ACC-1, and NOS terminator (Figure 1A). Kim et al (2015) provided the sequences of MON71800 event-specific element and ACC-1 gene while the sequences of CaMV35S promoter and NOS terminator were obtained from the National Center for Biotechnology Information (NCBI). The sequences of the whole insertion fragment containing a restriction enzyme recognize sequence between each gene as shown in Figures 1A and 1B. The full length of the pUC57-MON71800 is 3650 bp. The correctness of the insertion fragment was confirmed by ABI PRISM 3700 DNA analyzer (PerkinElmer, Boston, MA, USA). The copy number of pUC57-MON71800 was calculated using the following formula: $m = [n] [1.096 \times 10^{-21}$ g/copy] (Whelan et al., 2003) where n is the base pair of one copy of a plasmid or genome. Therefore, one plasmid molecule of pUC57-MON71800 (3650 bp) contained 4 ag of plasmid DNA.

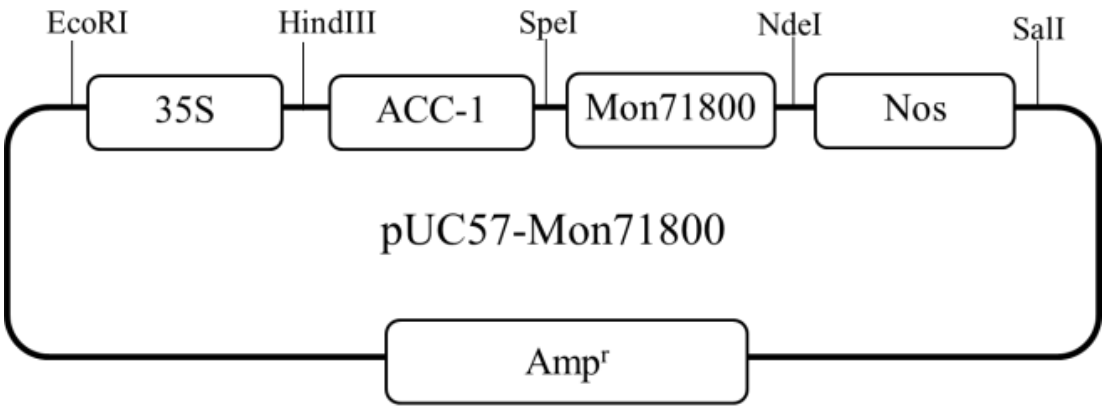

Figure 1 The schematic diagrams of the designed in-house pUC57-MON71800 which consist of CaMV35S promoter, ACC-1, MON71800 event-specific element, NOS terminator, ampicillin-resistant gene, and restriction recognize region between each gene.

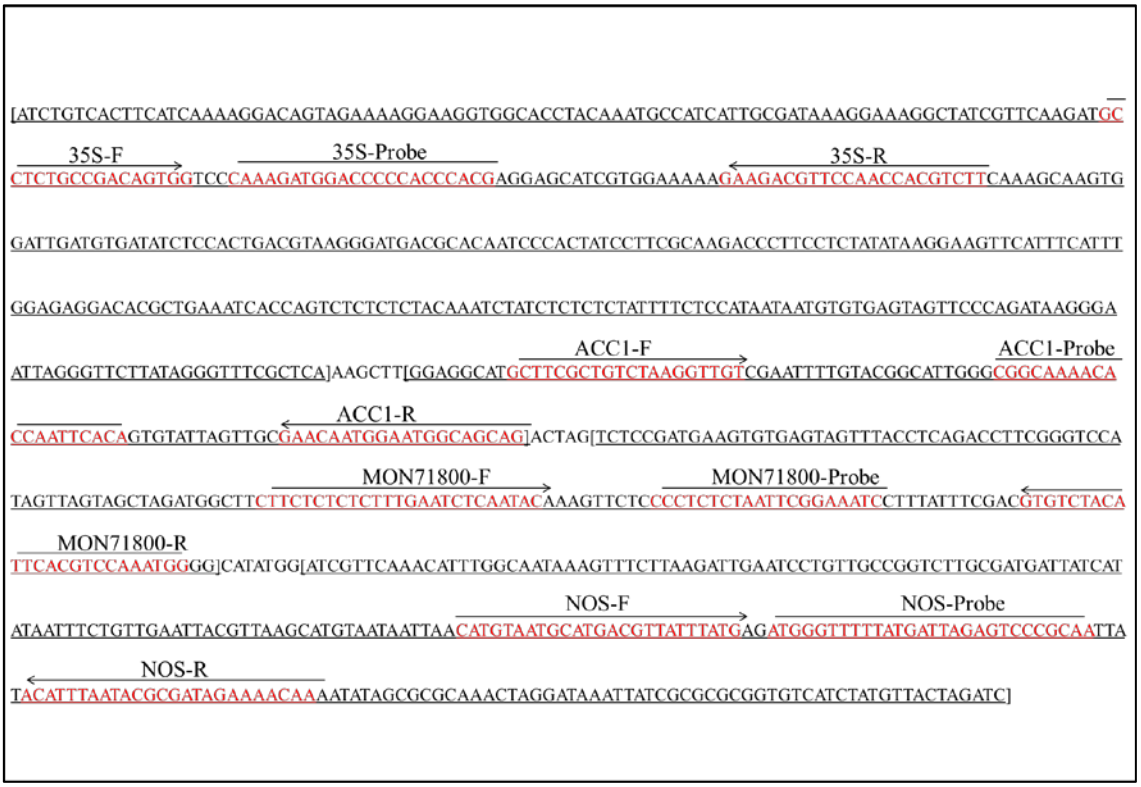

Figure 2 The fragment sequence of designed insertion genes. The red letter indicates the position of the forward primer, reverse primer, and probe of each gene.

3.2 Plasmid preparation

Constructed pUC57-MON71800 plasmid was transformed into One Shot TOP10 *E. coli* competent cells (Invitrogen™). The transformed cells were selected on an LB plate with 100 µg/ml ampicillin. The selected colony was confirmed by colony PCR with specific primer pair and cultured in 20 ml of shaking LB broth with 100 µg/ml ampicillin for 16 hours. The culture medium was collected and extracted for plasmid

using a plasmid extraction kit (Qiagen®). The quantification of plasmid was measured by spectrophotometer Multiskan GO (Thermo Fisher Scientific, Finland). The sequence of the prepared plasmid was confirmed by ABI PRISM 3700 DNA analyzer (PerkinElmer, Boston, MA, USA) before using it as a reference plasmid material.

3.3 DNA extraction

The sample of wheat and wheat's product was extracted by the in-house method named Lysis Method. 0.2 g of the sample was weighed and added Lysis buffer (Genescan, Eurofins) with 20 mg/ml Proteinase K 10 µl into a 1.5-ml tube. The sample was mixed by vortex mixture and incubated at 60°C for 1 hour. The supernatant was separated by centrifuge at 14,000 rpm for 15 min and added chloroform (1:1). The mixture of the supernatant and chloroform was mixed by vortex and centrifuged at 14,000 rpm for 15 min, and repeated twice. The clear upper layer was moved into a new tube and added Wizard Minipreps DNA purification resin (Promega Corporation, USA) (1:1). The molecule of the DNA and resin-bound was trapped on the filter of the column by Vac-Man® system (Promega Corporation, USA) and washed by cold 80% isopropanol. The column was moved into a new tube and centrifuged at 14,000 rpm for 2 min to remove the remaining isopropanol. Finally, 50 µl of warm sterile distilled water was added and waited for 10 min. The dissolved DNA was centrifuged at 14,000 rpm for 2 min and kept at -20°C until use. The extracted DNA was determined inhibitor in the PCR reaction by testing with ACC-1 amplification. 20 ng/µl of DNA was diluted in 1:4, 1:16, 1:64, and 1:256 and recorded crossing point (CP) for calculating ΔC extrapolated and R^2.

3.4 Primer and probe synthesis for Tetraplex Real-Time PCR

The sequences of the primers and probes of the MON71800 event-specific element and ACC-1 came after (Kim et al., 2015), following by the sequences of the CaMV35S promoter and NOS terminator (Waiblinger et al., 2008 & International Organization for Standardization, 2005). For the Tetraplex Real-Time PCR, four different probe colors need to be applied. Labeled probe and quencher on each gene as follows; CaMV35S promoter labeled 5'FAM-3'BHQ1, NOS terminator labeled 5'HEX-3'BHQ1, MON71800 labeled 5'TxRd-3'BHQ2, and ACC-1 labeled 5'Cy5- 3'BHQ3 (Table 1).

Table 1 The sequences of the oligonucleotide of primers and probes for detection of the CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 genes.

Target	Name	Sequence (5'-3')	Length	Reference
CaMV35S promoter	35S-F	GCCTCTGCCGACAGTGGT	18	International Organization for Standardization, 2005
	35S-R	AAGACGTGGTTGGAACGTCTTC	22	
	35S-P	FAM-CAAAGATGGACCCCCACCCACG-BHQ1	22	
NOS terminator	NOS-F	CATGTAATGCATGACGTTATTTATG	25	
	NOS-R	TTGTTTTCTATCGCGTATTAAATGT	25	
	NOS-P	HEX-ATGGGTTTTTATGATTAGAGTCCCGCAA-BHQ1	28	
MON71800	MON71800-F	CTTCTCTCTCTTTGAATCTCAATAC	25	Kim et al., 2015
	MON71800-R	CCATTTGGACGTGAATGTAGACAC	24	
	MON71800-P	TxRd-CCCTCTCTAATTCGGAAATC-BHQ2	20	
ACC-1	ACC1-F	GCTTCGCTGTCTAAGGTTGT	20	
	ACC1-R	CTGCTGCCATTCCATTGTTC	20	
	ACC1-P	Cyanine5-CGGCAAAACACCAATTCACA-BHQ3	20	

3.5 Tetraplex Real-Time PCR and LOD

The conditions of Tetraplex Real-Time PCR were optimized and compared with Simplex Real-Time PCR. The conditions of Simplex and Tetraplex Real-Time PCR are as follows; initial denaturation at 95°C for 10 min, denaturation at 95°C 5 min, annealing and extension at 60°C for 1 min for 40 cycles, and final cooling down at 40°C. The results of the crossing point (CP) were recorded. The conditions of both reactions are shown in Table 2.

Table 2 Reagents for detection of the CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 by Real-Time PCR (Simplex and Tetraplex).

Reagent	Final concentration/reaction (µM)				
	Simplex				Tetraplex
	CaMV35S promoter	NOS terminator	MON71800	ACC-1	
2xLightCycer Probe master mix	1x	1x	1x	1x	1x
35S-F	0.5	-	-	-	0.1
35S-R	0.5	-	-	-	0.1
35S-P	0.5	-	-	-	0.1
NOS-F	-	0.5	-	-	1.0
NOS-R	-	0.5	-	-	1.0
NOS-P	-	0.5	-	-	0.2
MON71800-F	-	-	0.5	-	0.1
MON71800-R	-	-	0.5	-	0.1
MON71800-P	-	-	0.5	-	0.1
ACC1-F	-	-	-	0.5	0.05
ACC1-R	-	-	-	0.5	0.05
ACC1-P	-	-	-	0.5	0.025
DNA template (ng)	50-100	50-100	50-100	50-100	50-200
Total reaction volume	20	20	20	20	20

To determine LOD of Tetraplex Real-Time PCR for detection of the in-house MON71800 reference plasmid, the concentration of the plasmid stock (10 ng) was examined and calculated to copy number. 10-fold serial dilution of plasmid was made and tested in the Tetraplex Real-Time PCR for 10 replications. The final dilution that could be detected over 95% will be the LOD of Tetraplex Real-Time PCR.

4. Results and Discussion

The specificity of primers and probes to pUC57-MON71800 plasmid

The Specificity of designed primers and probes were examined by the Simplex Real-Time PCR. The PCR reaction used the pUC57-MON71800 plasmid as a template DNA in different concentrations of 20, 10, and 5 ng/µl. The results showed a normal amplification curve in different crossing points (CP) (Table 4) and indicated that the designed primers and probes are specific to the CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1, which appear in the pUC57-MON71800 plasmid.

DNA extraction of wheat and wheat's product

To perform a screening test of MON71800 wheat, the optimization of DNA extraction of wheat and wheat's product is required. Wheat, Wheat flour, Instant noodle, and Cracker were employed as a testing matrix by using the in-house Lysis Method DNA extraction. The results of the DNA concentration showed a high concentration sufficient for PCR reaction (>20 ng/µl). The quality of the extracted DNA showed high purity (A260/280 =1.8-2.0). The inhibitor of the PCR reaction that appeared in the extracted DNA was determined by the inhibition test. ACC-1 primer and probe were used in the Simplex Real-Time PCR. The

inhibition test showed efficient PCR amplification with no inhibitor appeared (ΔC extrapolated < 0.5 and R^2 >0.98) (Table 3) (Wu et al., 2014), meaning that the in-house Lysis Method is appropriate for DNA extraction of wheat and wheat's product for using in Real-Time PCR reaction.

Table 3 Concentration, a ratio of A260/280, and inhibition test of the extracted DNA from wheat and wheat's products.

Sample	Concentration (ng/µl)	Ratio A260/280	Inhibition test	
			ΔC extrapolated	R^2
Wheat	5080.37±242.24	2.04±0.00	-0.23	0.99
Wheat flour	2155.22±264.58	1.95±0.00	-0.40	0.99
Instant noodle	1488.20±71.17	2.02±0.00	0.30	0.98
Cracker	1168.80±178.14	1.95±0.01	0.08	0.99

Comparison of Simplex and Tetraplex Real-Time PCR

The crossing points (CP) of the CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1, which use the PUC57-MON71800 as a template DNA in different concentrations of 20, 10, and 5 ng/µl, were compared between the Simplex and Tetraplex Real-Time PCR. The results showed that the Tetraplex Real-Time PCR has significantly lower CP than the Simplex Real-Time PCR, which indicates less PCR product or slower amplification in the Tetraplex Real-Time PCR (Table 4). The explanation is that the template of the reaction is plasmid DNA that appears very close between four target genes and interferes annealing of primers and probes to each target. Although the CP results showed a significant difference between the Simplex and Tetraplex Real-Time PCR by *t*-test, in qualitative screening detection, the PUC57-MON71800 can be used as a positive control. Besides, the Tetraplex Real-Time PCR can reduce cost and time as it can screen four target genes in only one reaction (Sint et al., 2012).

Table 4 Crossing point (CP) of CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 by using different concentrations of PUC57-MON71800 plasmid as a template in Simplex and Tetraplex Real-Time PCR reaction *$P < 0.05$ (t-test) (n=3).

Target	PUC57-MON71800 Concentration (ng/µl)	Crossing point (CP) ± SD		
		Simplex	Tetraplex	t-test
CaMV35S promoter	20	9.63 ± 0.37	10.31 ± 0.02	*
	10	10.80 ± 0.08	11.47 ± 0.09	*
	5	12.06 ± 0.35	12.77 ± 0.04	*
NOS terminator	20	9.78 ± 0.01	8.16 ± 0.15	*
	10	10.76 ± 0.14	8.86 ± 0.63	*
	5	12.35 ± 0.13	10.67 ± 0.23	*
MON71800	20	9.64 ± 0.35	10.94 ± 0.01	*
	10	10.30 ± 0.35	12.07 ± 0.12	*
	5	11.50 ± 0.16	13.32 ± 0.03	*
ACC-1	20	8.43 ± 0.06	9.40 ± 0.04	*
	10	9.46 ± 0.35	10.61 ± 0.12	*
	5	10.73 ± 0.09	11.89 ± 0.05	*

Limitation for detection PUC57-MON71800 plasmid by Tetraplex Real-Time PCR

To use the PUC57-MON71800 as a positive reference material, the Limitation of detection (LOD) is needed to be determined. A common definition of LOD is the lowest amount of DNA template that can be detected with a higher-than-stated percentage of confidence (95%) (Anonymous, 2011). In this regard, 10 replications of diluted PUC57-MON71800 at 1 pg and 0.1 pg were compared. All four target genes showed 100 % detectable at 1 pg but less than 95% at 0.1 pg, indicating that the LOD of the PUC57-MON71800 for

detection of CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 by the Tetraplex Real-Time PCR is 1 pg (Table 5).

Table 5 Limit of detection (LOD) of PUC57-MON71800 plasmid for detection of CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 in Tetraplex Real-Time PCR reaction.

Target	Replication	Ratio (Detect : Non-detect)		Limit of detection (LOD)
		1 pg	0.1 pg	
CaMV35S promoter	10	10:0	5:5	1 pg
NOS terminator	10	10:0	4:6	1 pg
MON71800	10	10:0	3:7	1 pg
ACC-1	10	10:0	7:3	1 pg

5. Conclusion

Only MON71800 is approved GM wheat in the ISAAA GM approval database. The problem of GM wheat screening tests is the contamination by other GM species such as corn, soybean, and canola during the harvesting and transportation. Therefore, Triplex Real-Time PCR of CaMV35S promoter, NOS terminator, and wheat endogenous ACC-1 is not adequate for identifying the GM wheat since the CaMV35S promoter and NOS terminator can also be found in other GM species. However, the Tetraplex Real-Time PCR can solve this problem. A perfect amplification of CaMV35S promoter, NOS terminator, MON71800 event-specific element, and ACC-1 indicates the appearance of MON71800 in a wheat sample but not in others. Due to the lack of MON71800 reference material in the commercial market, the in-house PUC57-MON71800 plasmid is necessary for use as a positive control. This research provides insertion sequences of four target genes, primers and probes sequences, Tetraplex Real-Time PCR conditions, and LOD of the in-house PUC57-MON71800 plasmid, which is useful and economizes the cost for the screening of the MON71800 GM wheat. Nonetheless, further studies on the identification of the contamination of GM corn, soybean, and canola in wheat samples are required.

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7. References

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