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Research paper

Effect of endogenous reference genes on digital PCR assessment of genetically engineered canola events

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ABSTRACT

Droplet digital PCR (ddPCR) has been used for absolute quantification of genetically engineered (GE) events. Absolute quantification of GE events by duplex ddPCR requires the use of appropriate primers and probes for target and reference gene sequences in order to accurately determine the amount of GE materials. Single copy reference genes are generally preferred for absolute quantification of GE events by ddPCR. Study has not been conducted on a comparison of reference genes for absolute quantification of GE canola events by ddPCR. The suitability of four endogenous reference sequences (*HMG-I/Y*, FatA(A), CruA and Ccf) for absolute quantification of GE canola events by ddPCR was investigated. The effect of DNA extraction methods and DNA quality on the assessment of reference gene copy numbers was also investigated. ddPCR results were affected by the use of single vs. two copy reference genes. The single copy, FatA(A), reference gene was found to be stable and suitable for absolute quantification of GE canola events by ddPCR. For the copy numbers measured, the *HMG-I/Y* reference gene was less consistent than FatA(A) reference gene. The expected ddPCR values were underestimated when CruA and Ccf (two copy endogenous Cruciferin sequences) were used because of high number of copies. It is important to make an adjustment if two copy reference genes are used for ddPCR in order to obtain accurate results. On the other hand, real-time quantitative PCR results were not affected by the use of single vs. two copy reference genes.

1. Introduction

Digital PCR is being widely used for the detection and quantification of genetically engineered (GE) events [1–3]. Specific and single copy endogenous reference genes are preferred for absolute quantification of GE events by PCR. Different endogenous reference genes have been used for real-time PCR detection and quantification of GE canola events. Five endogenous reference genes [acetyl-CoA carboxylase (*BnACCg8*), phosphoenolpyruvate carboxylase (*PEP*), oleoyl hydrolase (*FatA*)), high-mobility group protein I/Y (*HMG-I/Y*) and cruciferin A (*CruA*)] were compared for specific real-time PCR detection and quantification of *Brassica napus* [4]. Two different sequences were reported for the cloned fragments of *HMG-I/Y,* PEP and *CruA*, indicating the presence of genes in two copies [4]. On the other hand, *HMG-I/Y* was reported to be a single copy reference gene that can be used for quantification of GE canola events by real-time PCR [5]. It was also reported that the five endogenous reference genes mentioned above were not suitable for real-time PCR quantification of GE canola events as they were not specific between different species and also not stable among cultivars [4]. However, endogenous reference genes such as *CruA* and *HMG-I/Y* have been widely used for real-time PCR quantification of GE canola events and acceptable results were reported using the reference genes [examples: [6–8]. Recently, Acyl-ACP thioesterase (FatA(A)) gene was reported to be specific to the A genome of cultivated canola quality oilseed rape (*B. napus*, *B. rapa* and *B. juncea*) and recommended to be used as endogenous reference gene for real-time PCR [9]. There is no published information on the comparison of endogenous reference genes for absolute quantification of GE canola events by droplet digital PCR (ddPCR). The objectives of the study were to: 1. assess suitability of endogenous Cruciferin (Ccf), CruA, FatA(A) and HMG-I/Y reference genes for absolute quantification of GE canola events using ddPCR; 2. determine the influence of DNA extraction methods, DNA quality and cultivar variation on copy numbers of reference genes; and 3. compare the effect of reference genes on ddPCR and real-time PCR results.

Abbreviations: Ccf, Cruciferin; CruA, Cruciferin A; dPCR, digital PCR; ddPCR, droplet digital PCR; DMF, DNeasy® mericon Food kit; FatA(A), Acyl-ACP thioesterase; FID, Fast ID Genomic DNA extraction kit; GE, genetically engineered; GMO, genetically modified organism; GMQ2, GM Quicker II DNA extraction kit; *HMG-I/Y*, high-mobility group protein; NSF, NucleoSpin Food kit; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

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Table 1
DNA sequences of primers and probes and concentrations used for ddPCR and real-time PCR.

Name of target event or reference gene	Sequences (5' to 3')	concentration used for ddPCR (μM)	Concentration used for real-time PCR (μM)
GT73	F-CCA TAT TGA CCA TCA TAC TCA TTG CT	0.4	0.15
	R-GCT TAT ACG AAG GCA AGA AAA GGA	0.4	0.15
	P-FAM-TTC CCG GAC ATG AAG ATC ATC CTC CTT-BHQ1	0.2	
	P-FAM-TTC CCG GAC ATG AAG ATC ATC CTC CTT-TAMRA		0.05
HCN92	F-GTT GCG GTT CTG TCA GTT CC	0.4	
	R-CGA CCG GCG CTG ATA TAT GA	0.4	
	P-FAM-TCC CGC GTC ATC GGC GG-BHQ1	0.2	
OXY235	F-GAT AGA TGG TGG TGT GAG TCT TGT	0.4	0.3
	R-CCT AAC TTT TGG TGT GAT GAT GCT	0.4	0.3
	P-FAM-TGC CAT CAG CTG ACA CGC CGT GC-BHQ1	0.2	
	P-FAM-TGC CAT CAG CTG ACA CGC CGT GC-TAMRA		0.15
CruA	F-GGC CAG GGT TTC CGT GAT	0.2	0.2
	R-CCG TCG TTG TAG AAC CAT TGG	0.2	0.2
	P-HEX-AGT CCT TAT GTG CTC CAC TTT CTG GTG CA-BHQ1	0.2	
	P-VIC-AGT CCT TAT GTG CTC CAC TTT CTG GTG CA-TAMRA		0.2
FatA(A)	F-ACA GAT GAA GTT CGG GAC GAG TAC	0.3	0.3
	R-CAG GTT GAG ATC CAC ATG CTT AAA TAT	0.9	0.9
	P-HEX- AAG AAG AAT CAT CAT GCT TC-BHQ1	0.15	0.15
HMG	F-GGT CGT CCT CCT AAG GCG AAA G	0.2	0.2
	R-CTT CTT CGG CGG TCG TCC AC	0.2	0.2
	P-VIC-CGG AGC CAC TCG GTG CCG CAA CTT-BHQ1	0.2	0.2
Ccf	F-ATT GGG CTA CAC CGG GAT GTG T	0.2	
	R-GCT TCC GTG ATA TGC ACC AGA AAG	0.2	
	P-HEX-CGA TGG TGT CCC CAG TCC TTA TGT GCT C-BHQ1	0.2	

Table 2
Copy number variation of four endogenous references among three non-GE canola cultivars.

Canola cultivar (DNA source)	*HMG-I/Y copy numbers*[X,Y]	FatA(A) *Copy numbers*[X,Y]	CruA *copy numbers*[X,Y]	Ccf *copy numbers*[X,Y]	Comparison of reference means for each cultivar[Z]
Legend	6350 ± 217[c]	6083 ± 295[b]	11885 ± 462[b]	11852 ± 186[b]	[b, b, a, a]
Eagle	7283 ± 374[b]	6145 ± 192[b]	13369 ± 356[a]	13584 ± 460[a]	[b, b, a, a]
Parkland	8444 ± 149[a]	11764 ± 284[a]	13084 ± 552[a]	13397 ± 447[a]	[c, b, a, a]

10 ng DNA extracted with Fast ID DNA extraction method was used for ddPCR.
[X] Average of four ddPCR measurements plus minus standard deviation.
[Y] For each reference gene and copy numbers for the three cultivars, means assigned the same letter vertically are not significantly different (α = 0.05).
[Z] For each cultivar, reference means assigned the same letter horizontally are not significantly different (α = 0.05). For example, for Legend, means of *HMG-I/Y*, FatA(A), CruA and Ccf have the letters 'b', 'b', 'a' and 'a', respectively – means of CruA and Ccf were significantly higher than that of *HMG-I/Y* and FatA(A).

Table 3
Effect of four DNA extraction methods on the assessment of reference gene copy numbers.

DNA extraction method and cultivar	*HMG-I/Y copy numbers*	FatA(A) copy numbers	CruA copy numbers	Ccf copy numbers
FID – Legend	6841 ± 149[a]	6463 ± 43[a]	13741 ± 97[a]	13704 ± 304[a]
GMQ2 – Legend	4746 ± 234[b]	4662 ± 60[bc]	9641 ± 288[b]	9898 ± 252[b]
DMF – Legend	4673 ± 116[b]	4801 ± 31[b]	9221 ± 106[b]	9221 ± 276[c]
NSF – Legend	4738 ± 54[b]	4548 ± 127[c]	9198 ± 289[b]	9684 ± 137[bc]
FID – Eagle	7018 ± 202[a]	6550 ± 137[a]	13943 ± 578[a]	14417 ± 249[a]
GMQ2 – Eagle	4866 ± 89[b]	4610 ± 78[b]	9730 ± 193[bc]	9989 ± 246[b]
DMF – Eagle	4487 ± 228[b]	4526 ± 5[b]	8649 ± 296[c]	9009 ± 277[c]
NSF – Eagle	5018 ± 525[b]	4641 ± 93[b]	9963 ± 222[bc]	10560 ± 392[b]

FID = Fast ID DNA extraction kit; GMQ2 = GM Quicker II DNA extraction kit; DMF = DN*easy*® *mericon* Food kit; NSF = NucleoSpin Food kit. Average of three ddPCR measurements plus minus standard deviation. For each reference gene/cultivar and four DNA extraction methods (vertically), means assigned the same letter are not significantly different (α = 0.05). 10 ng DNA was used for ddPCR.

2. Materials and methods

2.1. Seed sources

Seeds of Armor BX (OXY235 canola event), Innovator (HCN92 canola event), Legend (non-GE canola), AC Parkland (non-GE certified canola) and 11canola cultivars (L120, InVigor® 5440, PV 533 G, V22-1, L159, L252, PV 530 G, 74-44 BL, L150, L156H and 1022 RR) were received from Oilseeds Program of the Grain Research Laboratory of the Canadian Grain Commission. The 11 canola cultivars were used to