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DIMETHYL SULFOXIDE IMPROVES SENSITIVITY AND SPECIFICITY OF RT-PCR AND QRT-PCR AMPLIFICATION OF LOW-EXPRESSED TRANSGENES

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Abstract – The expression of transgenes in a host plant may be low for a number of reasons. Both low expression and poor specificity of amplification were encountered during analysis of the expression of the *Arabidopsis* cytokinin oxidase/dehydrogenase *(AtCKX1)* gene in transgenic *Centaurium erythraea*. The optimization of the PCR protocol involved a gradient of annealing temperatures, as well as the application of seven PCR enhancers: formamide, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, trehalose, BSA and Tween-20. The best results for *AtCKX1* amplification were obtained at 55.1°C, with the addition of 5% DMSO. Glycerol and trehalose also improved the sensitivity of amplification, while formamide, ethylene glycol and BSA enhanced only the amplification of control purified targets, but not the transcripts. Tween-20 inhibited PCR. DMSO enhanced *AtCKX1* PCR amplification and improved the specificity of qPCR amplification, as well as the assay reproducibility. This work emphasizes the usefulness of additives, which are rarely used for PCR optimization in real-time experiments.

*Key words***:** Bovine serum albumin, dimethyl sulfoxide, ethylene glycol, formamide, glycerol, PCR additives, PCR enhancers, qPCR optimization, trehalose, Tween-20

INTRODUCTION

The generation of transgenic plants with a stable and high transgene expression is important for the success of crop improvement programs based on genetic engineering. Transgene expression is highly variable, even among clones transformed independently with the same construct, due to possible rearrangements of the exogenous DNA prior to integration, position effects, variation in transgene copy number and epigenetic factors such as DNA methylation (Meyer, 1998). Even strong constitutive promoters used in constructs, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or its enhanced versions, are susceptible to spatial and temporal regulation (van Leeuwen et al., 2001). Detecting the expression of transgenes introduced into host plants for which there is little or no sequence information presents a particular challenge, since there is no way to test *in silico* whether the designed primers are actually specific within the host genome context. If the transgene expression is very low, the amplification of its transcripts from complex cDNA mixtures can be outcompeted by the amplification of more abundant related host sequences, resulting in false negative reactions and unspecific amplification. In such cases, as well as for other problematic sequences, including targets with high %GC or samples with contaminations, extensive optimization of PCR protocols may be required. Optimization of Mg^{2+} concentration, buffer pH, denaturing and annealing times and temperatures, and cycle number is useful in some, but

not all cases, so the inclusion of appropriate PCR additive(s) in the amplification mix may be useful (Frackman et al., 1998). A variety of PCR additives and enhancers has been used to increase the specificity, yield or consistency of PCR reactions. PCR additives comprise a chemically heterogeneous group that includes amides, sulfoxides, sulfones, alkylammonium salts, compatible solutes, polyhydroxyl alcohols, proteins, nonionic detergents and nucleotide analogs (Table 1). The beneficial effects of additives are often template- and primer-specific and have to be determined empirically.

Among the most widely used PCR additives are the denaturants formamide and dimethyl sulfoxide (DMSO). They interfere with hydrogen bonding, thus facilitating strand separation, lowering DNA melting temperature (Tm) and consequently improving the specificity of primer binding (Sarkar et al., 1990; Bookstein et al., 1990; Varadaraj and Skinner, 1994; Frackman et al., 1998). Formamide can dramatically improve the specificity of PCR, but it also increases the efficiency of the amplification (Sarkar et al., 1990), probably due to the reduction of secondary structures. Acetamide showed an even better effect on PCR specificity, while 2-pyrrolodone had the greatest impact on PCR yield among different tested amides, especially for GC-rich targets (Chakrabarti and Schutt, 2001a). DMSO was proven to considerably enhance both the specificity and efficiency of DNA polymerization, not only driven by *Taq* (Bookstein et al., 1990; Baskaran et al., 1996; Kitade et al., 2003), but also by *Sequenase* (Winship, 1989), as well as *rTth* DNA polymerase that mediates both RT and PCR (Sidhu et al., 1996). Because of the potency of DMSO as a PCR enhancer, a number of other sulfoxides and sulfones have been tested (Table 1; Chakrabarti and Schutt CE, 2001b; 2002).

Long before the invention of PCR, it was shown that small tetraalkylammonium (TAA) ions can eliminate or even reverse the usual dependence of the DNA melting temperature on base composition (Melchior and Von Hippel, 1973). These additives preferentially bind to A-T pairs and stabilize them. Low concentrations of tetramethylammonium chloride (TMA-Cl) increase PCR efficiency and specificity (Hung et al., 1990; Chevet et al., 1995), while some other TMA salts (Table 1), especially TMA-oxalate, are even more potent (Kovárová and Dráber, 2000). Apart from the isostabilizing effect on DNA, TAA derivatives stabilize asymmetrical cyanine dyes such as SYBR Green I (SGI) in aqueous solutions, thus serving as potent enhancers of SGI-monitored qPCR (Shaik et al., 2008).

Betaine (N,N,N-trimethylglycine), a quaternary amine similar to TAA ions, is also an isostabilizing agent that equalizes the contribution of GC- and AT-base pairing to DNA stability by stabilizing AT-pairs while having a general denaturing effect (Rees et al., 1993). Betaine enhances the amplification of templates with varying GC content (Weissensteiner and Lanchbury, 1996; Baskaran et al., 1996; Henke et al., 1997), improves the processivity of "long and accurate" (LA) PCR (Hengen, 1997), aids the optimization of multiplex reactions (Hengen, 1997), broadens the optimal range for $MgCl_2$ concentrations, and allows the amplification of DNA samples of lower quality (Weissensteiner and Lanchbury, 1996). Another advantage of using betaine is that it acts as an osmoprotectant (compatible solute) and increases the resistance of *Taq* to denaturation (Hengen, 1997). Frackman et al. (1998) demonstrated by NMR analysis that the PCR enhancer provided by two commercial suppliers, *Qiagen* and *Clontech,* is in fact betaine. Disaccharide trehalose is another compatible solute and a kosmotropic agent that is effective in the thermostabilization of Taq (Spiess et al., 2004), but also renders thermolabile enzymes, including reverse transcriptases, restriction endonucleases and euthermal polymerases more resistant to thermal stress (Carninci et al., 1998; Mizuno et al., 1999). Moreover, trehalose protects *Taq* when freezing PCR master mixes (Klatser et al., 1998).

Several polyhydroxyl alcohols are also potent PCR enhancers (Table 1). Glycerol improves PCR specificity (Nagai et al., 1998; Varadaraj and Skinner, 1994). Ethylene glycol and 1,2-propanediol were found to be more effective than betaine in the amplification of 104 randomly selected GC-rich human

Table 1. Mode of action, application and suggested concentration of different types of PCR additives.

Type	Additive	Suggested final Conc.	Mode of action	Effects on PCR	Reference
Amides	Formamide	1-5% < 1 M	Disrupts base pairing	Increases specificity and efficiency Lowers Tm Reduces secondary structures	Sarkar et al., 1990 Varadaraj and Skinner, 1994 Kovárová and Dráber, 2000 Chakrabarti and Schutt, 2001a
	Acetamide	250 mM		Increases specificity and efficiency	Chakrabarti and Schutt, 2001a
	2-pyrrolidone	120 mM			
Sulfoxides	DMSO	1-10%	Disrupts base pairing	Improves specificity and efficiency, especially in GC-rich templates Lowers Tm Reduces secondary structures	Bookstein et al., 1990 Varadaraj and Skinner, 1994 Demeke and Adams, 1992 Baskaran et al., 1996 Sidhu et al., 1996 Frackman et al., 1998 Kitade et al., 2003 Jung et al., 2001
	Propyl sulfoxide	0.15 - 0.36 M		Improves specificity and efficiency	Chakrabarti and Schutt, 2002
	Methyl *sec*-butyl sulfoxide	0.03 – 0.3 M			
	Tetramethylene sulfoxide	0.28 - 1 M			
Sulfones	Methyl sulfone	0.57-1.15 M		Improves specificity and efficiency	Chakrabarti and Schutt, 2001b
	Sulfolane	0.15-0.5 M			
Tetraalkyl-ammonium salts	TMA-Cl	10 μM – 100 mM	Stabilizes A-T pairs Isostabilizes DNA Stabilizes SGI mixtures	Increases specificity and efficiency Increases PCR yield Enhances qPCR with SGI	Melchior and Von Hippel, 1973 Hung et al., 1990 Chevet et al., 1995 Kovárová and Dráber, 2000 Shaik et al., 2008
	TMA oxalate	2 mM		Increases specificity and efficiency	Kovárová and Dráber, 2000
	TMA hydrogen sulfate	0.5 – 50 mM			
	TEA-Cl	8 – 24 mM	Isostabilizes DNA Stabilizes SGI mixtures	Enhances qPCR with SGI	Melchior and Von Hippel, 1973 Shaik et al., 2008
	TPA-Cl	10 – 40 mM	Stabilizes SGI mixtures	Enhances qPCR with SGI	Shaik et al., 2008
	TPA-acetate	10 – 20 mM			
	TBA-Cl	8 - 16 mM			

Table 1. *Continued*

Type	Additive	Suggested final Conc.	Mode of action	Effects on PCR	Reference
Compatible solutes	Betaine	1-2 M	Isostabilizes DNA Facilitates strand separation Lowers Tm Weak thermostabilization effect on Taq	Improves PCR efficiency, specificity and processivity Increases the optimal range for $MgCl_2$ concentrations Increases qPCR efficiency	Rees et al., 1993 Baskaran et al., 1996 Weissensteiner and Lanchbury, 1996 Hengen, 1997 Henke et al., 1997 Frackman et al., 1998 Spiess at al., 2004
	Trehalose	0.2 M	Thermostabilization of Taq Decreases DNA Tm	Increases PCR yield Increases qPCR efficiency	Carninci et al., 1998 Mizuno et al., 1999 Spiess et al., 2004 Weissensteiner and Lanchbury, 1996
Polyhydroxyl alcohols	Ethylene glycol	1M	Decreases DNA Tm	Amplification of GC-rich sequences	Zhang et al., 2009
	Propylene glycol	0.8 M			
	Glycerol	5-10%		Increases efficiency Improves specificity	Varadaraj and Skinner, 1994 Nagai et al., 1998
Polymer	PEG 400	5-15%	Neutralizes contamination with plant polysaccharides		Demeke and Adams, 1992
Proteins	BSA	≤ 1 µg/µl	Scavenges contaminations	Relieves interference in PCR	Pääbo et al., 1988 Giambernardi et al., 1998 Kreader, 1996 Nagai et al., 1998
	T4 gene 32 protein	150 ng/µl	Scavenges contaminations Binds to ssDNA	Relieves interference in PCR	Kreader, 1996
Nonionic detergents	Tween-20	0.1-1%	Prevents secondary structure formation Neutralizes SDS contamination	Improves specificity and yield	Varadaraj and Skinner, 1994 Bachmann et al., 1990 Demeke and Adams, 1992 Gelfand and White, 1990
	Triton X-100	0.1-1%	Stabilizes *Taq* Neutralizes SDS contamination	Stabilizes enzymes	Liu et al., 1995 Gelfand and White, 1990
	Nonidet P-40	0.1-1%	Prevents secondary structure formation Neutralizes SDS contamination	Improves specificity and yield	Varadaraj and Skinner, 1994 Bachmann et al., 1990 Gelfand and White, 1990
Nucleotide analogs	dc^7GTP	dGTP: dc^7GTP =1:3	Lowers the Tm of the substituted DNA	Improves amplification of GC-rich DNA Destabilizes secondary structures	McConlogue et al., 1988 Multer and Boynton, 1995
	dITP		Lowers the Tm of the substituted DNA	Improves amplification of GC-rich DNA	Turner and Jenkins, 1995

DNA sequences with GC contents of 60-80% and lengths of 700-800 bp, without compromising PCR fidelity (Zhang et al., 2009).

The addition of bovine serum albumin (BSA) to PCR reactions is often beneficial for its ability to scavenge and neutralize a number of contaminants that inhibit *Taq*, including melanin (Giambernardi et al., 1998), hemin, iron chloride, tannic acids, fulvic acids, humic acid, extracts from feces, freshwater or marine water (Kreader, 1996), and other contaminants present in environmental or ancient samples (Pääbo et al., 1988). An ssDNA-binding protein, T4 gene 32 protein, was also effective in relieving interference in PCR when applied at lower concentrations than BSA (Kreader, 1996).

In some cases, nonionic detergents such as Tween 20, NP-40 or Triton X-100, can overcome amplification failure if applied at low concentrations (0.1-1%). They stabilize *Taq* (Liu et al., 1995), suppress the formation of secondary structures, reduce the loss of reagents through adsorption to tube walls and overcome inhibitory effects of trace amounts of SDS (Gelfand and White, 1990). NP-40 and Tween 20 enhance PCR specificity and yield (Bachmann et al., 1990; Varadaraj and Skinner, 1994). Triton X-100 applied as 0.4% was used for cell lysis, allowing the direct amplification of DNA from solid tissues without prior extraction (Liu et al., 1995).

Finally, base analogs 7-deaza-2'-deoxyguanosine 5'-triphosphate (dc7GTP) and deoxyinosine (dITP) that partially replace dGTP may be used for the amplification of templates with extremely high %GC and stable secondary structures (McConlogue et al., 1988; Multer and Boynton, 1995; Turner and Jenkins, 1995).

Some of the described additives may be used in various combinations (Baskaran et al., 1996; Nagai et al., 1998). Most of the additives have been reported to have beneficial effect on PCR amplifications as evaluated by end-point analysis (agarose electrophoresis followed by densitometric analysis). The efficiency of these agents in real-time PCR based on fluorescence detection from intercalating dyes is mostly unknown, with the exceptions of trehalose (Spiess et al., 2004), betaine (Spiess et al., 2004), DMSO (Jung et al., 2001) and TAA derivatives (Shaik et al., 2008), which were proven to enhance qPCR.

Both low expression and unspecific amplification of cDNA were encountered in the course of testing *Centaurium erythraea* Rafn. clones transformed with the *Arabidopsis* gene for the cytokinin oxidase/dehydrogenase isoform *AtCKX1*. Since the transformation was previously confirmed in all clones by means of genomic PCR with specific primers, the detection and quantification of *AtCKX1* expression, at least in some clones, was a matter of optimization of PCR and qPCR protocols. The aim of the current work was to optimize the PCR protocol for this transgene by combining a gradient of annealing temperatures with the application of seven different PCR enhancers: formamide, DMSO, glycerol, ethylene glycol, trehalose, BSA and Tween-20. The best combination of annealing temperature and additive was used for real-time quantification of the transgene expression.

MATERIALS AND METHODS

Plant material

For RNA extractions, the shoots (rosettes) and roots of the *in vitro* cultivated transgenic clones of *Centaurium erythraea* Rafn. were used. The *C. erythraea* plants were transformed using *Agrobacterium tumefaciens* GV3101 strain bearing binary vector pBinHTx-*AtCKX1*. The success of the transformation, i.e. the integration of the transgene into the genome, was confirmed by amplification of genomic DNA with *AtCKX1*-specific primers (Trifunović, unpublished data).

RNA extraction and reverse transcription

Total RNA was extracted from the shoots and roots of 4-week-old *AtCKX1*-transformed plants using the method described by Gašić et al. (2004). Prior to reverse transcription, the RNA samples were treated with DNAse I (Fermentas), following the manufac-

turer's protocol. The reverse transcription was performed on 1 μg total RNA, using GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems), with oligo-dT primers, according to the manufacturer's instructions.

Optimization of PCR for AtCKX1 using temperature gradient and different additives

Specific *AtCKX1* primers (forward: CAGCAGCAAACCTCAACGTG and reverse: ATCGCTAGAGGGTCGTAGGC) that amplify a 127-bp fragment were designed using Primer BLAST with *AtCKX1* GenBank sequence NM_12971. The primers were specific in the context of the *Arabidopsis* genome. The primers were checked for the eventual presence of internal secondary structures and the potential to form primer-dimers by NetPrimer software. The PCR mixtures were set in 25 μl, containing 2U of Fermentas *Taq* (EP0401), 10x Taq buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.3 μM primers, cDNA corresponding to 50 ng total RNA (isolated from *AtCKX1* clone 29), 0.5 μl dNTP mix (final 0.2 mM each) and 2.5 μl of 10x PCR additive or nuclease-free water. All additives were initially prepared as 10x stocks, to be used at the following final concentrations: formamide 2% (≈500 mM), DMSO 5%, glycerol 5%, ethylene glycol 1 M, trehalose 0.2 M, BSA 0.2 $\mu g\mu l^{-1}$ and Tween-20 0.2%. Plasmid *pBinHTx-AtCKX1*, digested with BamHI, was used as a positive control (2.5 ng/reaction). The cycling program included initial denaturation (95°C/10 min), followed by 40 cycles of denaturation (95°C/15 s) annealing at temperatures ranging from 49.1°C to 58.3°C for 30 s, extension (72°C/30 s) and final extension (72°C/10 min). The amplification was performed in the Thermal cycler PEQ STAR featuring temperature gradient. The PCR reactions (15 μl) were separated on large (24 x 20 cm) 2% agarose gels, at 120 V, using Biometra Compact L System and visualized using Quantum ST4 Gel Doc system.

Real-time PCR of AtCKX1 transgene

Real-time PCR reactions were set using Maxima™ SYBR Green/ROX Master Mix (Fermentas), with cDNA corresponding to 50 ng RNA, with or without the addition of 2.5 μl 50% DMSO (5% final). The reactions that contained DMSO had 2.5 μl less water, so that all reactions had a final volume of 25 μl. Plasmid *pBinHTx-AtCKX1* was digested with BamHI to be used as the qPCR standard in serial dilutions (5 ng, 1 ng, 500 pg, 100 pg and 10 pg). All reactions were performed in triplicate. The qPCR program was the same as for PCR, but with annealing at 56.5°C, followed by melting curve analysis. The melting curve analysis was performed by cooling the reactions to 60°C and then increasing the temperature to 95°C with a slope of $0.1°Cs^{-1}$, while measuring the fluorescence continuously. The amplification was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems Co., Foster City, USA). The results were analyzed and presented using 7000 System SDS Software.

RESULTS AND DISCUSSION

Effect of different PCR additives on the amplification of AtCKX1 transcripts from total cDNA

In order to investigate the effects of overexpression of the CKX enzyme on cytokinin metabolism and *in vitro* morphogenesis, *C. erythraea* plants were transformed with the *AtCKX1* isoform from *Arabidopsis*. Even though the integration of the foreign gene into the host genome was successful in 12 clones, as confirmed by genomic PCR (unpublished data), the *AtCKX1* expression was not detectable in any of the clones using standard PCR reagents and the cycling program described in Materials and Methods at the calculated annealing temperature of 54°C (data not shown). Varying the annealing temperature in a range from 49.1°C to 58.3°C gave no amplification products (Fig. 1; water). Since the amplification of the control plasmid DNA (*pBinHTx-AtCKX1*) at mid-point gradient T of 54°C gave the product of the expected length of 127 bp, it was concluded that the amplification conditions were adequate, providing that a sufficient template is present. In a previous work, qPCR amplification of both *AtCKX1* and *AtCKX2* cDNAs from transgenic potato (*Solanum tuberosum* L. cv. Désirée) was suc-

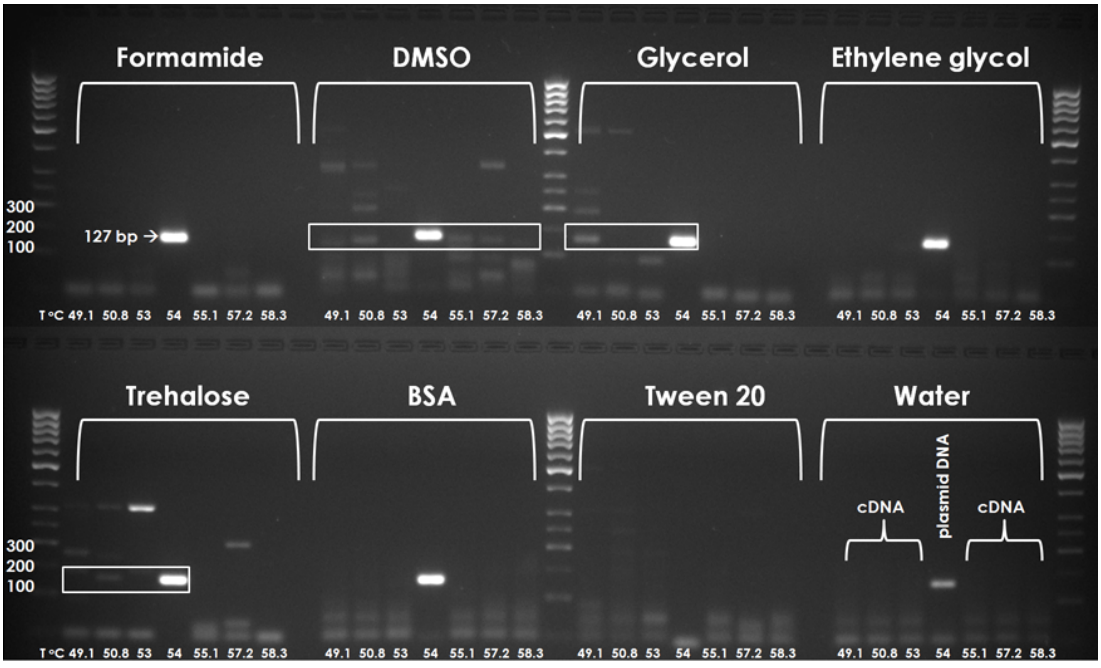

Fig. 1. PCR optimization of AtCKX1 amplification by gradient of annealing temperatures and seven different additives analyzed on a single gel for comparison. Either 100 ng of total cDNA or 2.5 ng of digested plasmid DNA were amplified in 25 µl-PCR reactions, of which 12.5 µl were loaded per lane.

cessful and specific at the annealing temperature of 57°C (Raspor et al., 2012). The conditions for amplification of the *AtCKX1* transcript from 50 ng of total cDNA were then optimized by applying the same temperature gradient with seven annealing temperatures (of which the mid-point T was used for positive control), but in the presence of seven different PCR enhancers or water, giving a total of 48 test reactions (indicated as cDNA, Fig. 1) and 8 positive control reactions (indicated as plasmid DNA). Each enhancer was applied in one concentration, based on available literature data (Table 1), as follows: formamide 2% (final), DMSO 5%, glycerol 5%, ethylene glycol 1 M, trehalose 0.2 M, BSA 0.2 $\mu g \mu l^{-1}$ and Tween-20 0.5%.

Of the seven tested PCR enhancers, all but Tween-20 significantly improved the PCR efficiency (yield) of the control plasmid DNA (Fig. 1) in comparison to the reaction with water. Tween-20 has been previously reported to enhance PCR specificity and yield (Bachmann et al., 1990; Varadaraj and Skinner, 1994). In addition, this nonionic detergent reverses the inhibitory effects on *Taq* of some plant polysaccharides that may be present as contaminants (Demeke and Adams, 1992), so it should be suitable for the amplification of DNA from plant samples. However, in our system, even plasmid DNA failed to amplify in the presence of Tween-20.

The amplification of the control plasmid DNA was much more efficient in the presence of formamide, ethylene glycol or BSA in comparison to amplification without any additives. As discussed earlier, formamide is a widely used denaturant that improves PCR efficiency and the specificity of primer annealing (Sarkar et al., 1990; Bookstein et al., 1990; Varadaraj and Skinner, 1994). Ethylene glycol destabilizes the high melting region of polypeptide-bound DNA and reduces the extent of higher-ordered structure in chromatin (Zhang et al., 2009), meaning that this additive should be more useful for the amplification of genomic DNA fragments rather than cDNA. In addition, it appears that ethylene glycol is able

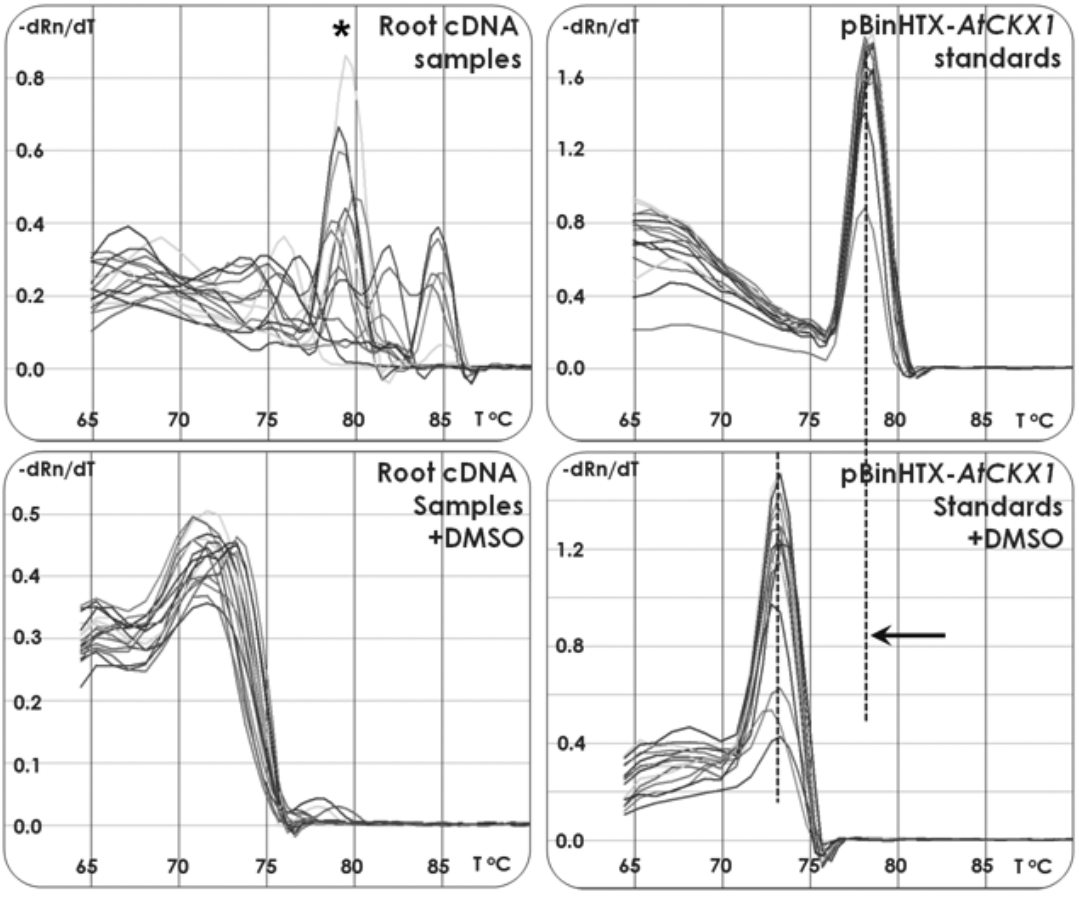

Fig. 2. Effect of DMSO on specificity of *AtCKX1* amplification. Melting curves of amplification products of *AtCKX1* cDNA samples (left panels) and the corresponding plasmid standards (*pBinHTX-AtCKX1,* right panels) in the absence and presence of 5% DMSO are shown. Each graph shows multiple curves, representing either a group of samples, e.g. different *AtCKX1* clones in 3 replicates, or a set of standards in serial dilutions, also in 3 replicates. The effect of DMSO on lowering the melting temperature is indicated by an arrow. In the absence of DMSO, the melting curves for the root *AtCKX1* samples are rather erratic, with peaks corresponding not only to the specific product (labeled with an asterisk), but also to a number of unspecific products. The *AtCKX1* amplification with DMSO is entirely specific, with a single peak. The graphs are screenshots of melting curve analysis results generated by 7000 System SDS Software.

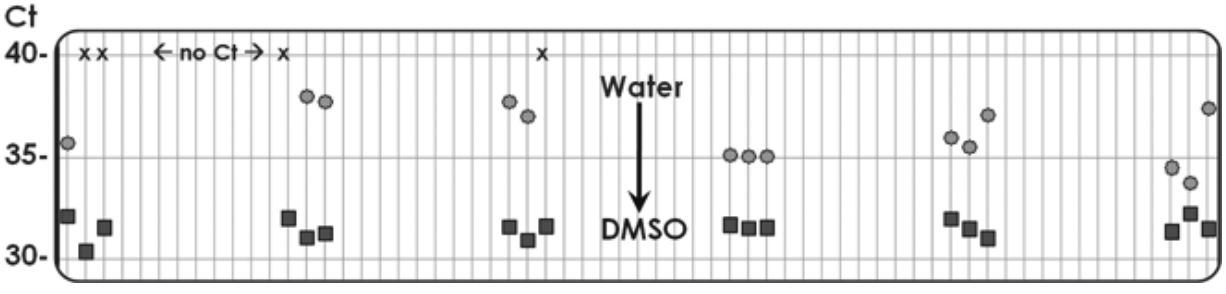

Fig. 3. Effect of DMSO on efficiency and reproducibility of *AtCKX1* amplification. A set of six *AtCKX1* samples (clones), each in 3 replicates, was amplified in standard qPCR reactions (circles) or with addition of 5% DMSO (squares). The absence of amplification (no Ct) is indicated as "x". The image was generated by merging screen shots of "Ct vs. well position" representations generated by 7000 System SDS Software.

to modulate the annealing process of GC-rich templates (Zhang et al., 2009), but the GC content of the *AtCKX1* amplicon was not an issue, being as low as 44%. Finally, BSA is not only a scavenger for contaminants (Kreader, 1996), but also a general stabilizing agent, so its addition in some cases allows specific fragment amplification from a complex mixture (Nagai et al., 1998). However, despite their different modes of action, these agents failed to support the amplification of *AtCKX1* cDNA.

Finally, at certain annealing temperatures and in the presence of DMSO, glycerol or trehalose, the expected amplification product of 127 bp was observed for cDNA samples (Fig. 1). With the addition of trehalose, the specific amplification band was present only at annealing temperatures <54°C. Trehalose also lowers DNA melting temperature (Tm) and therefore increases PCR efficiency, especially for GC-rich templates (Spiess et al., 2004). Even though trehalose thermostabilizes *Taq* polymerase, at higher temperatures it destabilizes primer binding, i.e. lowers annealing T (Spiess et al., 2004), which might explain its failure to assist *AtCKX1* amplification above 54°C. The addition of glycerol in some cases allows the specific fragment amplification from complex mixtures (Nagai et al., 1998). Glycerol-water mixtures have a lower dielectric constant, resulting in reduced DNA Tm in solutions with DNA-stabilizing Na^+ and Mg^{2+} ions (Sorokin et al., 1997), which is likely the reason why glycerol was effective only at lower annealing temperatures, much like trehalose. However, even at lower annealing temperatures, neither glycerol nor trehalose ensured complete specificity of the reaction, since in both cases unspecific products were also present.

DMSO was the only additive that allowed amplification of *AtCKX1* over a range of annealing temperatures from 49.1 to 57.2°C, and, more importantly, almost completely eliminated unspecific amplification at 55.1°C, as shown in Fig. 1. The remaining low-MW unspecific bands still present at 55.1°C are probably primer leftovers and primer-dimers formed because the amount of the template was insufficient. Several modes of action have been suggested for the PCR enhancement with DMSO, including the elimination of nonspecific amplification, changing the thermal activity profile of DNA polymerase and destabilizing the intramolecular secondary structures within the template for efficient annealing of primers (Sidhu et al., 1996). Because DMSO performed best among the tested additives in the end-point assay, it was used for the quantification of *AtCKX1* cDNA by real-time PCR.

Effect of DMSO on sensitivity and specificity of AtCKX1 qPCR amplification

The specificity of a PCR reaction is easily visualized upon electrophoretic separation and ethidium-Br staining of the products. The specificity of a qPCR reaction is estimated by melting curve analysis of the reaction product(s) that follows the cycling program – the samples are first cooled and then gradually heated with continuous monitoring of the fluorescence. For single-target samples with a high initial copy number, the SGI fluorescence declines linearly with increasing temperature, followed by a steep decline in fluorescence as the specific product melts (Ramussen et al., 1998). This transition or melting temperature (Tm) can be presented as a single sharp peak if negative first derivative of fluorescence is plotted vs. temperature increase (-dRn/dT). As can be seen in Fig. 2, the purified target (plasmid standards in serial dilutions) has a sharp peak at 78.6°C, indicating its Tm. The addition of DMSO lowers the Tm of the amplicon to 73.3°C (indicated by an arrow in Fig. 2), which is consistent with the previously discussed denaturing effects of DMSO.

The amplification of *AtCKX1* cDNAs with Maxima™ SYBR Green/ROX Master Mix according to Fermentas' protocol was not specific, even though this Master Mix contains an optimized buffer that in our hands worked well with many other targets. As shown in Fig. 2, the melting curves of amplification products for 12 *AtCKX1* clones (root cDNA, all in triplicates) have multiple peaks corresponding to both specific and nonspecific amplification products. The specific products can be identified because they have the same Tm as the correspond-

ing plasmid standards (as indicated in Fig. 2 with an asterisk). The samples of shoot cDNA had similar profiles as root cDNA samples (data not shown). In the presence of DMSO in the reaction mixtures, the amplification of *AtCKX1* transcript was entirely specific, with a single melting peak matching the melting point of *pBinHTX-AtCKX1* standards. However, the melting curves for samples were somewhat broader in comparison to the standards. The melting curves of low-copy number samples, even if their templates are purified (and not a complex cDNA mixture as in our experiments) are much broader and often shifted toward lower temperatures in comparison to the same samples of high-copy number (Ramussen et al., 1998). This is because the primer dimers and non-specific products, which are preferentially produced in the low-copy number samples, tend to melt at lower temperatures and over a broader range (Ramussen et al., 1998). It should be noted that the specificity of the amplification with DMSO was better at 56.5°C (Fig. 2) than at 55.1°C (data not shown), meaning that the optimization of the annealing T based on PCR cannot be directly applied to qPCR, probably due to differences in the composition of the Master Mixes.

Effect of DMSO on efficiency and reproducibility of qPCR amplification of low-copy targets

In addition to improving specificity, the presence of DMSO in qPCR reactions also enhanced the efficiency and reproducibility of the assay. This is illustrated in Fig. 3, where the threshold cycle (Ct) values for six *AtCKX1* clones (root samples, in triplicates) obtained in standard reactions are compared to Ct values of the same samples amplified in the presence of DMSO. If amplified without DMSO, some of the samples had no detectable product at all (no Ct, indicated as "x" in Fig. 3). However, in the presence of DMSO, all samples were successfully amplified, with significantly lower Ct values, meaning that the amplification efficiency was improved. Moreover, the differences among the biological replicates of the same sample in the presence of DMSO were very small, indicating improved reproducibility of the assay.

Jung et al (2001) have also demonstrated the usefulness of DMSO application in the qPCR quantification of rare transcripts. They used two different qPCR kits, both based on SGI detection, and the highest amplification rates were found by adding 5% DMSO to the reaction mixtures in both kits. Under these conditions, the low-expressed gene (MT1-MMP) amplification product increased ≈ 6-fold compared to the experiments without the addition of DMSO (Jung et al., 2001). The amplification was drastically reduced above a DMSO concentration range of 5%, while no specific product was detectable at 10% DMSO, probably due to an inhibition of *Taq*. Because of the ability of DMSO to increase the sensitivity and specificity of qPCR to detect very low expressed genes, the authors proposed the addition of 5% DMSO as an additive in ready-to-use reaction mixtures for real-time PCR with SGI. Our results strongly corroborate their findings.

CONCLUSIONS

It can be concluded that formamide, DMSO, glycerol, ethylene glycol, trehalose and BSA were effective as PCR enhancers that increased the yield of reactions with control plasmid *AtCKX1* DNA samples, while Tween 20 completely inhibited PCR for an unknown reason. However, only DMSO, glycerol and trehalose improved the PCR sensitivity so as to allow for amplification of the same target from complex cDNA mixture. While glycerol and trehalose were effective only at lower annealing temperatures and did not eliminate nonspecific amplification, DMSO enabled amplification of the *AtCKX1* transcript at a range of annealing temperatures, providing for specific amplification at 55.1°C. When applied in qPCR mixtures containing SGI, 5% DMSO decreased the melting temperatures of the amplicons and increased the specificity, sensitivity, efficiency and reproducibility of the assays.

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Abbreviations: *BSA – bovine serum albumin; CKX - cytokinin oxidase/dehydrogenase; Ct – threshold cycle ; dc7GTP - 7-deaza-2'-deoxyguanosine 5'-triphosphate; dITP – deoxyinosine; DMSO - dimethyl sulfoxide; qPCR – quantitative PCR; RT– reverse transcription; SDS – sodium dodecyl sulfate; SGI – SYBR Green I; TAA – tetraalkylammonium; TBA – tetrabutylammonium; TEA – tetraethylammonium; Tm – DNA melting temperature; TMA – tetramethylammonium; TPA – tetrapropylammonium*