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Short Communication

Differential expression of cellulose synthase (CesA) gene transcripts in potato as revealed by QRT-PCR

Olawole O. Obembe*, Evert Jacobsen, Jean-Paul Vincken [1], Richard G.F. Visser

Laboratory of Plant Breeding, Wageningen University, Box 386, 6700AJ Wageningen, The Netherlands

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Two transgenic potato lines, csr2–1 and csr4–8 that contained two different antisense cellulose synthase (CesA) genes, csr2 and csr4, respectively were crossed. The aim, amongst others, was to investigate the possibility of generating double transformants to validate a hypothetical presence of the proteins of the two CesA genes in the same cellulose synthase enzyme complex. SYBR-Green quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) assays were carried out on four CesA gene transcripts (CesA1, 2, 3, and 4) in the wild type genetic background, and on the two antisense CesA gene transcripts (CesA2 and 4) in the progeny resulting from the cross between the two transgenic potato lines. The quantitative RT-PCR analyses revealed different expression patterns of the two CesA genes. The CesA2 mRNA was shown to be relatively more abundant than CesA4 mRNA, regardless of the genetic background, suggesting that the two proteins are not present in the same enzyme complex.
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1. Introduction

The quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) is a precise, sensitive, flexible and simple procedure, which has grown to become the method of choice for the detection and quantification of mRNA [1,2]. Hence, the increasing applications of the technology in forensics and molecular medicine, including cancer and HIV researches [3–5], functional genomics and biotechnology [6–8]. The quantitative RT-PCR that employs the use of nonspecific double-stranded DNA (dsDNA) binding fluorophores, such as SYBR Green, is particularly used extensively for quantifying gene transcripts from plant cells [2].

Persson et al. [9] used transcript expression analyses in addition to genetic crossing to identify two *Arabidopsis* CesA proteins, CesA2 and CesA6, as close relatives, and also to confirm that they are incorporated into the cellulose synthase complex subunits at different developmental stages of the plant. It is generally believed that the cellulose synthase complexes that assemble primary and secondary cell walls are each composed of three distinct CesA catalytic subunits (for reviews, see [10,11]). The speculation that the potato CesA2 and CesA4 are members of the same cellulose synthase enzyme complex [12] has sparked off a line of investigation on the possibility of generating double transformants, through genetic crossing, with the combined effects of the antisense constructs of the two CesA genes. One of the objectives of this investigation was to validate the above speculation. However, various analyses carried out on the tubers of the progeny have consistently revealed more pronounced morphological, cellular and cell wall phenotypes in the single csr2 transformant tubers than in the double csr2/csr4 transformants ([13,14], unpublished data), suggesting that the two potato CesA proteins may not be members of the same enzyme complex, as one would expect more severe phenotypes of the double transformants, should the two CesA genes be present in the same complex.

This study explores the use of quantitative real-time RT-PCR as a tool to confirm this likelihood. The paper provides evidence that suggests that the potato CesA2 and CesA4 proteins are indeed not present in the same complex.

2. Results and discussion

2.1. Relative expression of four potato CesA genes in the potato plant

Relative expression of four potato CesA mRNAs in various tissues of the potato plant was examined using quantitative SYBR-Green Reverse Transcriptase-mediated (RT) PCR, an assay which allows

Abrreviations: CesA, cellulose synthase; RT-PCR, reverse transcription polymerase chain reaction.

* Corresponding author at: Present address: Department of Biological Sciences, College of Science and Technology, Covenant University, PMB 1023 Ota, Ogun State, Nigeria. Tel.: +234 8060164341.
E-mail address: obembe@covenantuniversity.com (O.O. Obembe).
[1] Present address: Department of Food Sciences, Wageningen University, Bomenweg 2/8129, 6700EV Wageningen, The Netherlands.

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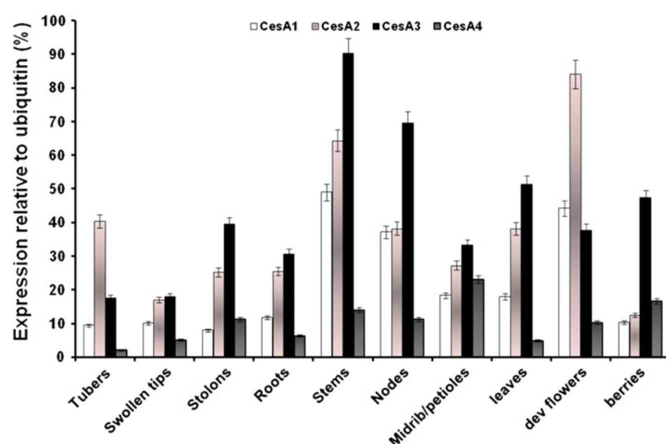

Fig. 1. SYBR-Green Real-time RT-PCR analysis of CesA genes in various tissues of the wild type potato plant. RNA levels for each were expressed relative to the amount of ubiquitin RNA and multiplied by 100.

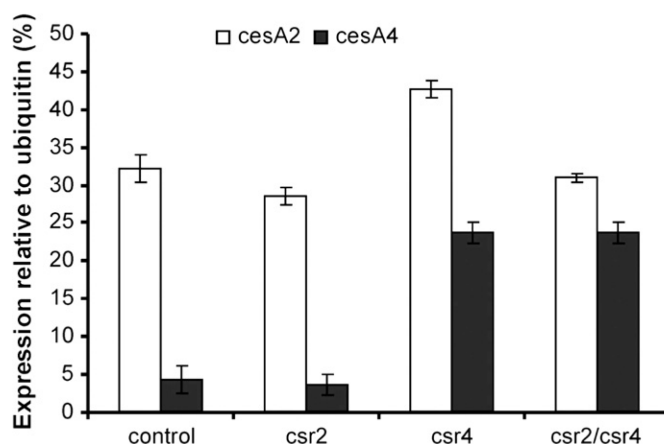

Fig. 2. SYBR-Green Real-time RT-PCR analysis of CesA2 and CesA4 genes in the tubers of the offspring. RNA levels were expressed relative to the amount of ubiquitin RNA, multiplied by 100.

even weakly expressed genes to be accurately quantified [15]. The relative abundance of CesA2 and CesA3 mRNAs was high in most tissues used for the expression analysis (Fig. 1). The CesA3 mRNA's relative abundance is highest in the stems (90%), followed by the nodes ($\approx$70%). The CesA2 mRNA was most highly expressed in the developing flowers (>80%), followed by the stems (>60%). The relative abundance of CesA1 and CesA4 mRNAs was intermediate and low, respectively, in most tissues (Fig. 1). Of particular interest was the relative abundance of CesA2 and CesA4 mRNA levels in the potato tuber. It was observed that CesA2 mRNA level (40%) was relatively more abundant in the tuber than that of CesA4 mRNA (<5%), probably hinting at the relative importance of the CesA2 protein in complex assembly in the tuber. Hence, our speculation that this critical function of the CesA2 protein may have been interfered with by the presence of the antisense CesA2 gene construct, and as such may be responsible for the various phenotypes exhibited by the crs2 tubers ([13,14]; unpublished data). This assumption further motivated the transcript expression analysis for the CesA2 and CesA4 genes in the tubers of the progeny.

2.2. Expression analysis for CesA2 and CesA4 in the tubers of the offspring plants

The relative expression of CesA2 and CesA4 mRNAs in the tubers of the four clones of the offspring plants was examined using quantitative SYBR-Green RT PCR. The analysis confirmed previous expression analysis that the relative abundance of the CesA2 mRNA was higher than that of CesA4, irrespective of the genetic background. This indicates that the two proteins are most probably not co-expressed and as such cannot be present in the same complex, since co-expressing CesA proteins are expected to be present in more or less equimolar amount for them to interact together and assemble the same cellulose synthase enzyme complex. Similar evidence was reported by Persson et al. [9], who confirmed that the *Arabidopsis* CesA2 and CesA6 proteins were not members of the same cellulose synthase complex but were incorporated into the complex at different developmental stages of the plants.

Fig. 2 shows the similar expression of CesA2 in the csr2 and the double csr2/csr4 clones but a higher expression in the csr4 clone. The analysis indicated general up-regulation of the CesA genes in the presence of the csr4 construct. It may be that down-regulation of the CesA4 has triggered compensatory mechanism that resulted in the forced synthesis of other closely related CesA genes. The SYBR-Green RT-PCR assay has been adjudged to be extremely sensitive for quantifying transcript expression among members of large gene families [16], as such the above observations are presumably fair depictions of the transcript levels in the various genetic backgrounds.

Nevertheless, medium-level down-regulation of the CesA2 mRNA was observed in the csr2 tubers. This is, however, contrary to our expectation for high-level down-regulation, which was premised on earlier observations of marked phenotypes in the csr2 tubers ([13,14], unpublished data). We had initially been tempted to attribute these phenotypes to the influence of the antisense CesA2 construct on the supposed central role of the CesA2 protein in complex assembly, in the potato tuber. However, with the compelling evidence of down-regulation of primary wall CesAs and Cellulose synthase-like (Csl) genes by the naturally occurring small-interfering RNA (siRNAs) derived from the HvCesA6 transcript expression in barley [17], the phenotypes may more likely be the result of down-regulation of other CesA genes by siRNAs and not the result of down-regulation of CesA2. Validating this possibility in future research therefore seems very interesting to pursue.

3. Conclusion

The QRT-PCR analyses of the CesA gene transcripts in the wild type and in the offspring obtained from the genetic crossing of the two antisense potato lines, revealed different expression patterns of the CesA genes. In particular, CesA2 mRNA is relatively more abundant than CesA4 mRNA in most tissues, and especially in the tuber. These results indicate that the proteins of the potato CesA2 and CesA4 genes are not present in the same enzyme complex.

4. Materials and methods

4.1. Plant material and growth conditions

The potato (*Solanum tuberosum* Linn., cultival kardal) plants, csr2–1 and csr4–8, used for the crosses carried in antisense orientation, csr2 sequence of the potato CesA2 gene (accession number AY221089) and the csr4 sequence of the CesA4 gene (accession number AY221089), respectively [12]. Expression of the antisense constructs was targeted to the tuber by using a granule bound starch synthase promoter to drive its expression.

Tubers obtained from the two antisense potato plants [12] were grown and cross-pollinated as reported by Obembe et al. [13].

Table 1
Overview of the SYBR-Green Primers used. Primers for the CesA genes were based on the class specific regions of the corresponding CesA.

Primer	Gene	Sequences (5′–3′)
CSR1-F	CesA1	CAGCCCTCATGCCTCAGATAA
CSR1-R	CesA1	AAATACCGGTGATTGGCCAA
CSR2-F	CesA2	TGAGGCAGATTTGGAGCCA
CSR2-R	CesA2	GACCCACCACAACAGCTCTTC
CSR3-F	CesA3	CGGCTGTTTTTGTTGCTTCA
CSR3-R	CesA3	CGATTGAGGAACACCACCATT
CSR4-F	CesA4	TCGAGGAAGGAATCGAAGGA
CSR4-R	CesA4	GCGGCATGAGGGAAGCTT
UBI3-F	Ubiquitin	TTCCGACACCATCGACAATGT
UBI3-F	Ubiquitin	CGACCATCCTCAAGCTGCTT

4.2. Quantitative SYBR-Green RT-PCR analysis for four potato CesA genes

Gene-specific primers were developed for four CesA genes (CesA1, 2, 3 and 4) and for ubiquitin (as internal control) using the Primer Express software (version 1.5, PE Applied BioSystems, CA, USA). Sequences of all the primers used are shown in Table 1. The tissues used for analysis were: tubers, swollen tips, stolons, roots, stem, nodes, midribs/petioles, leaves, developing flowers and berries. Total RNA was isolated from 3 g (fresh weight) of the different tissues as described elsewhere [18]. Reverse transcription reaction and SYBR-Green PCR were performed as described below. For first strand cDNA synthesis, 1 μg of total RNA was treated with 0.5 μl DNAse I RNase free (10 U/μl; Invitrogen) and incubated with 5 μl of 10× Taqman RT buffer, 11 μl of 25 mM $MgCl_2$, 10 μl of 10 mM dNTP mix, 2.5 μl of 50 μM random hexamer primers, 1.0 μl RNAse inhibitor (20 U/μl) and H_2O until a final volume of 39 μl for 30 min at 37 °C and 5 min at 75 °C. The mixture was then incubated for 10 min at 25 °C and 30 min at 48 °C with 1 μl of MultiScribe reverse transcriptase (50 U/μl; Applied Biosystems). The reaction was then terminated by heating the sample for 5 min at 95 °C. Aliquots of 50 ng of cDNA were used in SYBR-Green PCR analysis according to the manufacturer's protocol on the ABI PRISM7700 sequence detection system (Perkin-Elmer Applied Biosystems) with the primers listed in Table 1. Relative quantification of the CesAs RNA expressions was performed using the comparative C_T method according to the User Bulletin No. 2 (ABI PRISM7700 sequence detection system; Perkin-Elmer Applied Biosystems). The differences in Ct_t values, called ΔC_t, between the CesA mRNA and endogenous ubiquitin control mRNA were calculated in order to normalize the differences in the cDNA concentrations for each reaction. RNA expression level was expressed as percentage of the control RNA expression level using the equation $2^{-\Delta Ct_t} \times 100\%$.

4.3. Quantitative SYBR-Green RT-PCR analysis for CesA2 and CesA4 in the tubers of the offspring plants

The same procedure as described above was used for RNA isolation and the SYBR-Green RT-PCR analysis. The analysis was done with young, freshly harvested tubers of about the same developmental stage. The gene-specific primers of CesA2 and CesA4 genes as well as that of the ubiquitin were used to analyse their expression in the tubers of the four clones; single csr2 transformant, single csr4 transformant, double csr2/csr4 transformant and the control plant.

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