

Full Length Research Paper

Offspring of the crosses of two anti-sense potato plants exhibit additive cellulose reduction

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Two transgenic potato lines, *csr2-1* and *csr4-8*, containing two different antisense genes: *csr2* and *csr4*, respectively, were crossed to investigate the possibility of achieving reduction in cellulose content in the tuber cell walls of the progeny. The progeny containing both transgenes (double *csr2/csr4* transformant) exhibited reduction of 63% in cell wall cellulose content, while the single transformants *csr2* and *csr4* had a 44 and 22% reduction in cellulose content, respectively.

Key words: Cellulose content, cellulose synthase, double transformant, genetic crossing, *Solanum tuberosum*.

INTRODUCTION

Cellulose deposition in the cell wall plays a vital role in controlling cell growth which in turn influences the final morphology of plant organs. Crystalline cellulose microfibrils are formed in the cell wall by spontaneous association of, probably 36, β -D-glucan (cellulose) polymers synthesized by plasma membrane-bound cellulose synthase (CesA) complexes (rosettes), which are believed to comprise six subunits (Delmer and Amor, 1995). Each of the complex subunits is conceived to have at least six CesA proteins to be able to synthesise 36 glucan chains (Brown and Saxena, 2000). There is a body of evidence that indicates that each subunit of the cellulose synthase complex comprises at least three distinct CesA isoforms (Gardiner et al., 2003; Taylor et al., 2003; Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Robert et al., 2004).

The strategy of generating double mutants through genetic crossing was used to identify two *Arabidopsis* CesA proteins, CesA2 and CesA6, as close relatives, which can interchangeably substitute for one other in a subunit (Persson et al., 2007a). The authors reported that the double mutants did not exhibit significant decrease in cellulose as compared with the single mutants, hence indicating that both proteins are not components of the same subunit. Transcript expression analyses of these genes confirmed the above evidence, in that the CesA genes exhibited different expression patterns, suggesting that they are incorporated into the complex subunits at different developmental stages of the plant. Also a comparison of their sequences revealed that they share

very high sequence identity at the protein level (Persson et al., 2007a). In a similar study, the same group (Persson et al., 2007b) generated double mutants from two *Arabidopsis* mutants that are deficient in xylan synthesis and observed that the double mutants had significantly less xylose compared with the single mutant plants.

In this study, a cross was made between two transgenic antisense potato lines, *csr2-1* and *csr4-8*, whose tuber cell walls exhibit low levels of cellulose as compared to the wild type. The objective was to investigate the possibility of achieving further reduction in cellulose content in the tuber cell walls of the progeny. This paper reports the cellulose content reduction in *csr2/csr4* double transformants.

MATERIALS AND METHODS

Plant material and growth conditions

Potato (*Solanum tuberosum* Linn.) plants used for the crosses carried, in antisense orientation, *csr2* sequence of the potato CesA2 gene (accession number AY221089) or the *csr4* sequence of the CesA4 gene (accession number AY221089) as earlier reported (Obembe et al., 2008). Expression of the antisense constructs was targeted to the tuber by using a granule bound starch synthase (gbss) promoter to drive its expression.

Pollen of the plant line *csr4-8* was used to fertilize plant line *csr2-1* to produce berries (12). A total of 488 seeds were removed from the berries, dried and prepared for germination. 100 seedlings, representing the different genotypes, were grown in soil in the

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' to 3')
CSR1-F	<i>CesA1</i>	CAGCCCTCATGCCTCAGATAA
CSR1-R	<i>CesA1</i>	AAATACCGGTGATTGGCCAA
CSR2-F	<i>CesA2</i>	TGAGGCAGATTTGGAGCCA
CSR2-R	<i>CesA2</i>	GACCCACCACAACAGCTCTTC
CSR3-F	<i>CesA3</i>	CGGCTGTTTTTGTTCCTTCA
CSR3-R	<i>CesA3</i>	CGATTGAGGAACACCACCATT
CSR4-F	<i>CesA4</i>	TCGAGGAAGGAATCGAAGGA
CSR4-R	<i>CesA4</i>	GCGGCATGAGGGAAGCTT
UBI3-F	Ubiquitin	TTCCGACACCATCGACAATGT
UBI3-F	Ubiquitin	CGACCATCCTCAAGCTGCTT

greenhouse under 3,000 lux and in a light/dark period of 16/8 h.

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

The real-time reverse transcription polymerase chain reaction (RT-PCR) is the method of choice for the detection of mRNA (Bustin, 2000). Gene-specific primers were developed for four *CesA* genes (*CesA* 1, 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 (Kuipers et al., 1994). 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 x Taqman RT buffer, 11 µl of 25 mM MgCl₂, 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 H₂O 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 *CesA*s 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 C_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 $2^{-\Delta C_T} \times 100\%$.

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 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 *csr2/4* transformant and the control plant. The analysis was done with young, freshly harvested tubers of about the same developmental stage.

Isolation of cell wall material and analysis of the cellulose content

One tuber from three representatives of the four clones from the offspring were pooled and ground to a fine powder in liquid nitrogen. For each isolation, 10 g of this tuber material was extracted in a 50 mM Tris[HCl], pH 7.2 solution containing 1% SDS for 3 h at room temperature with continuous shaking. The CWM was spun down by centrifugation at 4600 rpm for 30 min. Subsequently, the residue was washed with water, ethanol, acetone and air-dried. 50 mg of this CWM was then incubated for 90 min at 120°C in 5 ml 2M trifluoroacetic acid (TFA). The remaining cellulose was spun down and obtained as pellet, which was washed with water and ethanol. The pellet was suspended in 67% (v/v) H₂SO₄. The suspension was heated gently and diluted appropriately to determine the cellulose content colorimetrically using anthrone as a colouring agent according to (Updegraff, 1969). Acid hydrolysis of cell wall materials and colorimetric assays were performed in quadruplicate and standard deviations were calculated accordingly. Cellulose content in the cell walls was expressed as percentage (w/w) of cellulose per total 'crude' CWM (without removal of the starch).

RESULTS AND DISCUSSION

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 that is widely believed to be the method of choice not only for the detection of mRNA (Bustin, 2000) but also for the quantification of mRNA (Udvardi et al., 2008). The assay also allows even weakly expressed genes to be accurately quantified (Pfaffl et al., 2002). The relative abundance of *CesA2* and *CesA3* mRNAs was high in most tissues used for the expression analysis, whereas the relative abundance of *CesA1* and *CesA4* mRNAs was intermediate and low, respectively, in most tissues (Figure 1A). Of particular interest was the relative abundance of *CesA2* and *CesA4* mRNA levels in the potato tuber. It was observed that *CesA2* mRNA level was relatively more abundant in the tuber than that of *CesA4* mRNA. This probably explains the observed more severe cellular phenotypes in the *csr2* tuber (Obembe and Vincken, 2008).

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

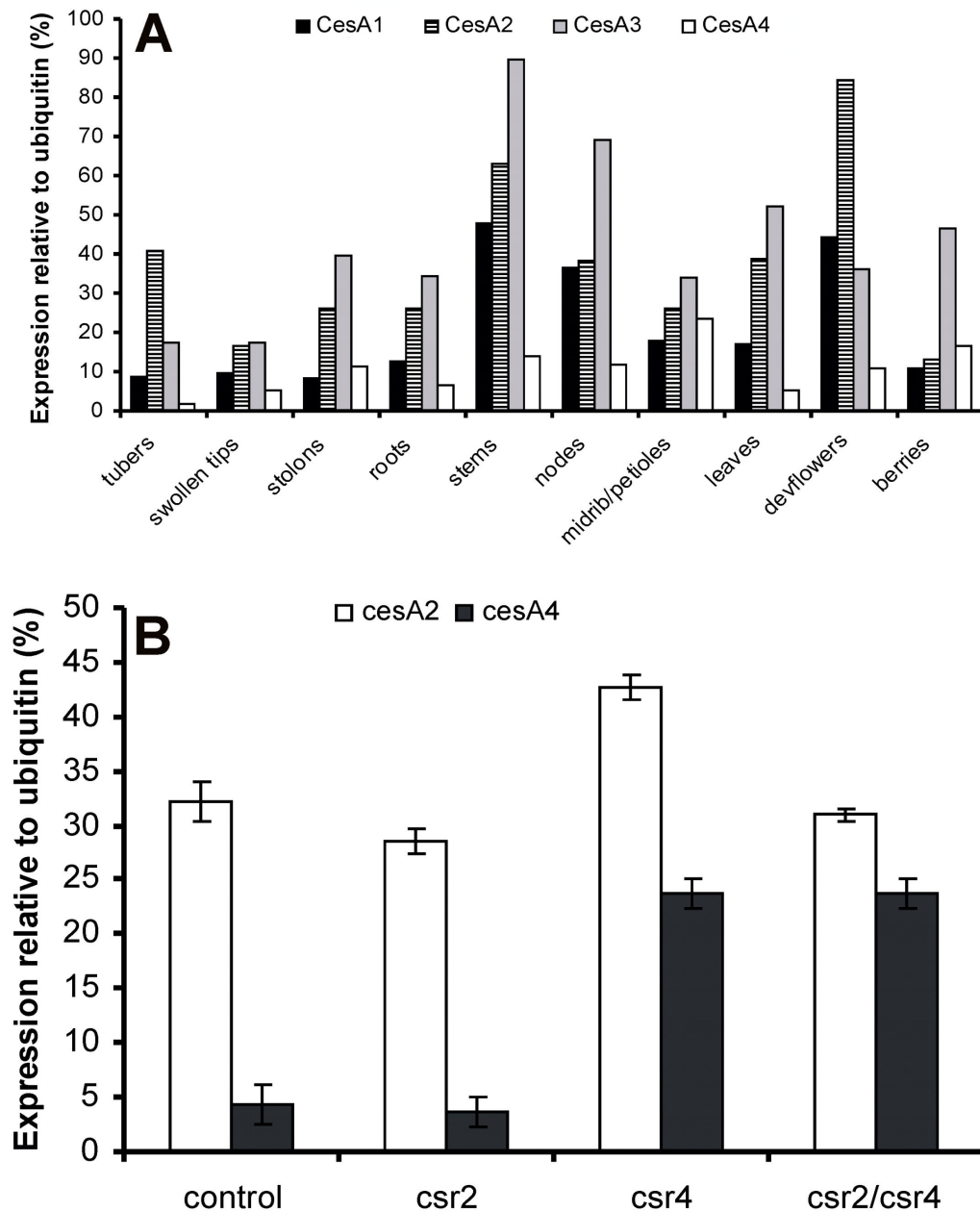


Figure 1. SYBR-Green Real-time RT-PCR analysis of CesA genes. Transcript expression analysis of CesA genes in various tissues of the wild type potato plant. (A) Transcript expression analysis of CesA2 and CesA4 genes in the offspring; (B) RNA levels for each were expressed relative to the amount of ubiquitin RNA and multiplied by 100.

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.

Figure 1B showed 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 CesA genes in the presence of the *csr4* construct. This notion was corroborated by

similar up-regulation of CesA4, in the *csr4* and the double *csr2/csr4* clones. It may be that down-regulation of the CesA4 has triggered up-regulation of other closely related CesA genes. Though this event can compensate for the down-regulated gene, it does not guarantee that the products of the newly triggered CesA genes will assemble into cellulose synthase complex. The SYBR-Green RT-PCR assay has been adjudged to be extremely sensitive for quantifying transcript expression

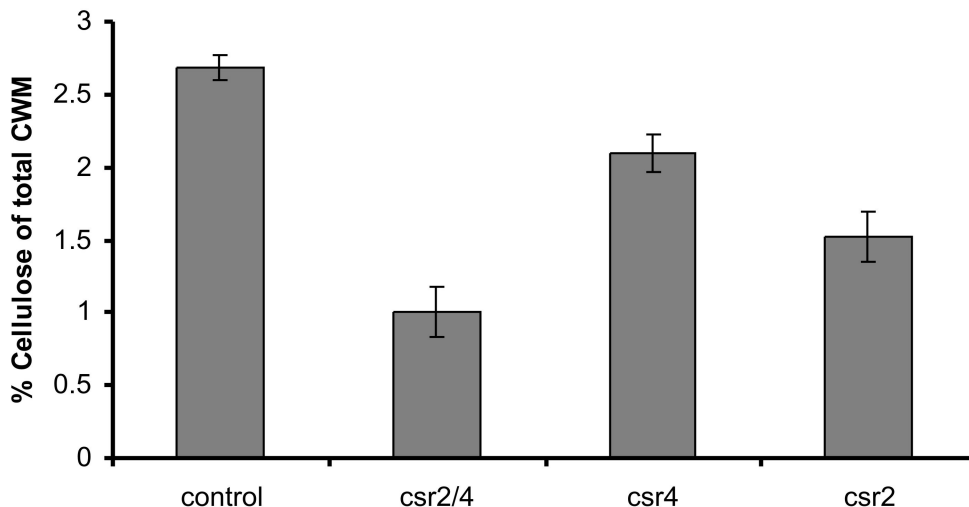


Figure 2. Cellulose content in the offspring tubers. Cellulose content of tuber material was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material (including starch). All measurements were performed as quadruplicate and standard deviations are represented by the bars.

among members of large gene families (Czechowski et al., 2004), as such the above observations are presumably fair depictions of the transcript levels in the various genetic background.

The apparently medium level of down-regulation of the *CesA2* mRNA can be argued against for not being strong enough to justify the phenotype in the *csr2* tubers. However, this observation might be explained by the possibility of cross-amplification of other *CesA* sequences in the potato genome, which may have influenced the results. To verify this possibility, the two sets of primers were used for *csr2* and *csr4*, in standard PCR, using plasmid DNAs of the four *csr* sequences as templates. It was observed that both sets could still amplify the other three *CesA*s sequences to a minor extent (data not shown). The problem of cross-amplification could not have been guarded against better than it was. The primers were designed such that they are as specific to their respective gene sequences as they can possibly be.

Cellulose reduction was observed in transformed tuber cell walls

Compared to the control, which was 2.7% of the total CWM (Figure 2) there was a 63% cellulose reduction in the *csr2/csr4* double transformant (1% of total CWM), whereas the single transformants *csr2* (1.5% of total CWM) and *csr4* (2.1% of total CWM) had a 44% and 22% reduction in cellulose content, respectively. This is in agreement with the report of Lane et al. (2001) that showed additional reduction in cellulose production in the double mutant *rsw2-1 rsw1* of the *Arabidopsis* relative to both single mutants *rsw2-1* and *rsw1*. Similar additive

reduction in lignin content was observed in tobacco double transformant that resulted from the crossing of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD down-regulated transgenic tobacco lines (15).

The 22% reduction in cellulose content of the *csr4* progeny tubers, however, contradicts an earlier report of 60% cellulose reduction in the single *csr4* - 8 (parent) transformants (Oomen et al., 2004). The authors remarked that the cellulose content of the *csr4* transformant series was not correlated to the Principal Component Analysis cluster plot, a discrepancy, which might explain our conflicting results. One had expected that the double transformants would display the most severe cellular phenotypes (Obembe and Vincken, 2008), owing to their low level of cellulose content. The question as to why the double transformant tubers are not exhibiting similar features as the single *csr2* transformant might be explained by the possibility of a hindrance to complex assembly as a result of the presence of the two antisense constructs in one background. Even though this implies that less cellulose microfibrils are produced in the double transformants, the ones that are produced might be normal, and as such there may be no impact on morphology. One is left to speculate therefore that factors other than reduction in crystalline cellulose content might be important in bringing about structural and morphological changes.

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