African Journal of Plant Science Vol. 4(12), pp. 474-478, December 2010
Available online at http://www.academicjournals.org/AJPS
ISSN 1996-0824 ©2010 Academic Journals

*Full Length Research Paper*

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

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Accepted 30 October, 2008

**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 | 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 |

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 $MgCl_2$, 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 values, called $\Delta C_t$, 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 $2^{-\Delta Ct}$ x 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 trifloroacetic 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) $H_2SO_4$. 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

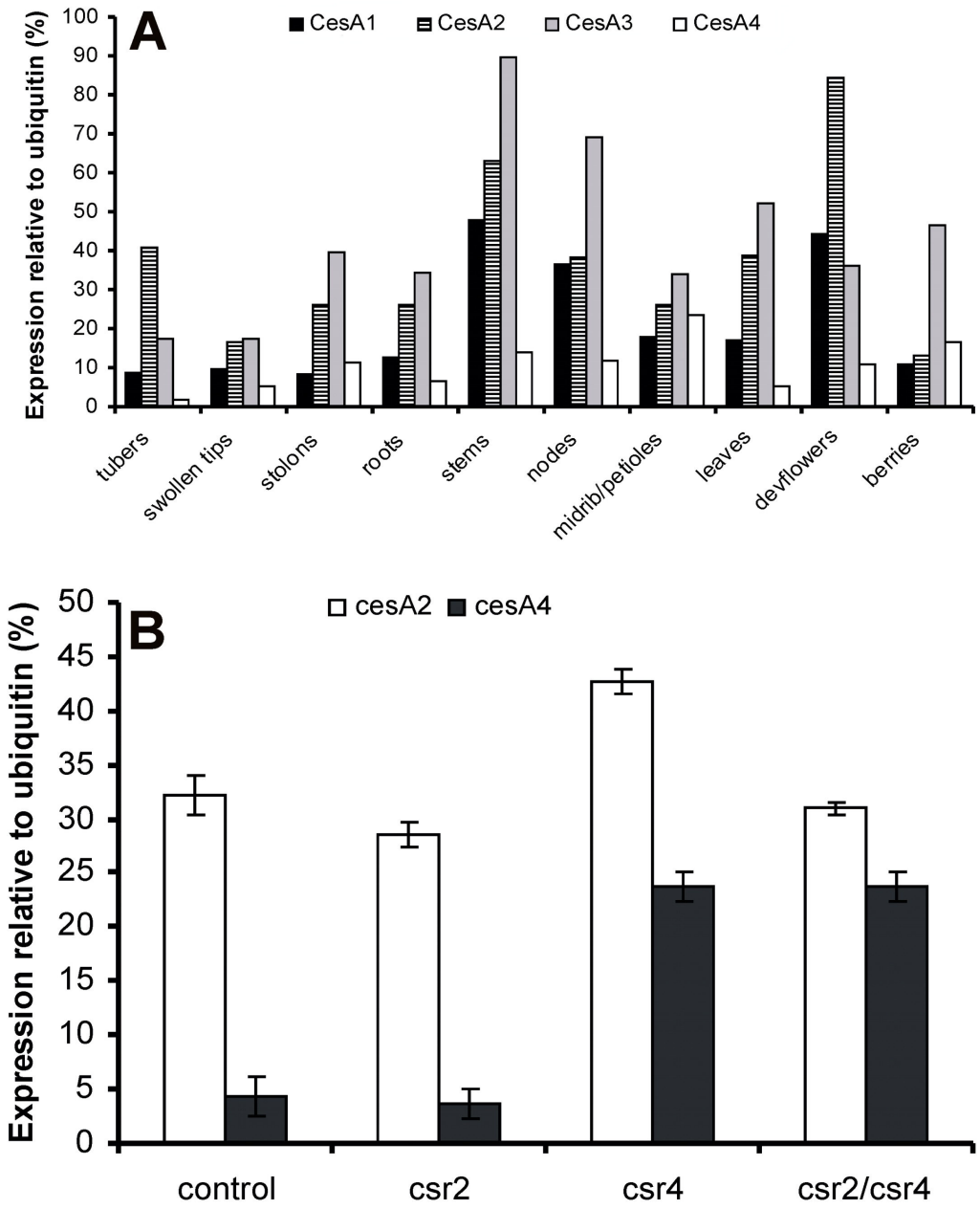


**Figure 1.** SYBR-Green Real-time RT-PCR analysis of CesA genes. Transcript expression analysis of CesA genes in various tissues of the wild type potato plant. (A) Transcript expression analysis of CesA2 and CesA4 genes in the offspring; (B) RNA levels for each were expressed relative to the amount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

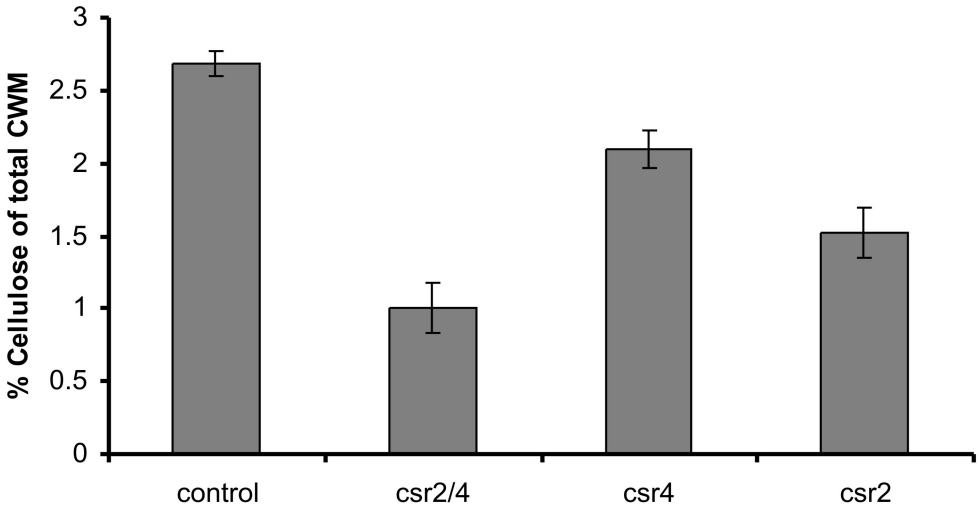


**Figure 2.** Cellulose content in the offspring tubers. Cellulose content of tuber material was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material (including starch). All measurements were performed as quadruplicate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

## ACKNOWLEDGEMENTS

This work was supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) The

Netherlands and by a grant from the Laboratory of Plant Breeding, Wageningen University. The author would like to thank Jean-Paul Vincken and Richard Visser for their assistance.

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