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# Modulation of the cellulose content of tuber cell walls by antisense expression of different potato (*Solanum tuberosum* L.) *CesA* clones

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**Abstract**

Four potato cellulose synthase (*CesA*) homologs (*StCesA1*, *2*, *3* and *4*) were isolated by screening a cDNA library made from developing tubers. Based on sequence comparisons and the fact that all four potato cDNAs were isolated from this single cDNA-library, all four *StCesA* clones are likely to play a role in primary cell wall biosynthesis. Several constructs were generated to modulate cellulose levels in potato plants in which the granule-bound starch synthase promoter was used to target the modification to the tubers. The *StCesA3* was used for up- and down-regulation of the cellulose levels by sense (SE-*StCesA3*) and antisense (AS-*StCesA3*) expression of the complete cDNA. Additionally, the class-specific regions (CSR) of all four potato cellulose synthase genes were used for specific down-regulation (antisense) of the corresponding *CesA* genes (csr1, 2, 3 and 4). None of the transformants showed an overt developmental phenotype. Sections of tubers were screened for altered cell wall structure by Fourier Transform Infrared microspectroscopy (FTIR) and exploratory Principal Component Analysis (PCA), and those plants discriminating from WT plants were analysed for cellulose content and monosaccharide composition. Several transgenic lines were obtained with mainly decreased levels of cellulose. These results show that the cellulose content in potato tubers can be reduced down to 40% of the WT level without affecting normal plant development, and that constructs based on the CSR alone are specific and sufficient to down-regulate cellulose biosynthesis.


*Keywords:* Antisense; Cellulose; Cell wall biosynthesis; Gene family; Potato; Specific knockout

## 1. Introduction

The identification of the first plant *CesA* gene by Pear et al. (1996) permitted the characterization of a large multigene family. Many studies have focussed on the identification of the specific functions of the individual *CesA* family members to explain the requirement for their large number. These studies include phylogenetic characterisation of the different genes, expression profiling, but also analysis and comparison of *Arabidopsis* plants harbouring a mutation in the different *CesA* genes (Fagard et al., 2000; Holland et al., 2000; Richmond, 2000; Vergara and Carpita, 2001). A number of primary cell wall mutants in various of the 10 family members in *Arabidopsis* have been identified in various screens for developmental phenotypes: *rsw1* (*AtCesA1*), *ixr1* (*AtCesA3*), and *ixr2* allelic to *prc* (*AtCesA6*) (Arioli et al., 1998; Desprez et al, 2002; Fagard et al., 2000;

*Abbreviations:* *CesA*, Cellulose synthase catalytic subunit A; CSR, Class Specific Region; CWM, cell wall material; FTIR, Fourier Transform InfraRed; PC, Principal Component; PCA, Principal Component Analysis.

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Scheible et al., 2001). The cellulose-deficient phenotypes obtained by mutation in single *CesA* genes has led to a hypothesis that the cellulose synthase may be a heteromeric complex. *Arabidopsis* mutants with defects in cellulose deposition in secondary walls have also been obtained: *irregular xylem* locus, *irx1*, *irx3* and *irx5*, all encode *CesA* homologs (Turner et al., 2001). Taylor et al. (2003) have subsequently demonstrated a physical interaction between *irx1* and *irx3*, further supporting the hypothesis that multiple *CesA* proteins can be present in one cellulose synthase complex. Additionally, the classification of CesA proteins based on sequence homology of the so-called Class Specific Region (CSR) showed that the primary and secondary cell wall related CesA proteins group in separate clusters (Vergara and Carpita, 2001). Nevertheless, the exact function of the different *CesA* proteins in one complex is still unclear.

The mutant approach constitutes one strategy to study the function of different *CesA* family members, and the composition of the cellulose synthase complex. However, this approach is difficult to apply in most crop plants, where an additional biotechnological goal is control of cellulose content. Therefore, in species such as potato, the use of transgenic approaches is favoured. Additionally, the antisense expression of CesA genes in *Arabidopsis thaliana* has shown to result in a slightly different phenotype in comparison with the phenotype of plants with a mutation in the corresponding gene (Burn et al., 2002). The modulation of *CesA* RNA expression levels and concomitantly cellulose content in crop plants has been demonstrated in tobacco (Burton et al., 2000). In this study, we have focussed on the introduction of altered cellulose levels in potato by antisense and over-expression of a complete potato *CesA* gene. In addition, we have used the class specific region (CSR) of four potato *CesA* genes in antisense constructs to specifically reduce the expression levels of individual *CesA* genes.

## 2. Results

### 2.1. PCR strategy and screening of a potato cDNA library

A primer pair based on the U1 and U2 regions of *CesA* sequences from *Acetobacter xylinum*, cotton and rice was developed to amplify the plant conserved region (P-CR, Pear et al., 1996) of a potato *CesA* cDNA. The 500-bp amplified fragment was used to screen a cDNA library derived from mRNAs isolated from swelling stolon tips, resulting in the isolation of four different potato *CesA* clones. *StCesA3* (AY221087) is full length, but *StCesA1* (AY221086), *2* (AY221089) and *4* (AY221088) lack up to 1 kb of the 5′ region.

### 2.2. Relationship of the potato CesA sequences to other plant CesAs

The derived amino acid sequences of the potato class-specific regions (CSR) were compared with sequences from *Arabidopsis*, cotton, maize and poplar. Since three of the four potato *CesA* clones were not full length, sequence comparisons were based only on the CSR. This region is important in revealing sequence divergence and for the clustering of different *CesA* genes from different plant species (Vergara and Carpita, 2001). The four potato *CesA* proteins group in three different clusters of the dendrogram (Fig. 1). The seven *CesA* sequences *AtCesA4/7/8*, *GhCesA1/2* and *PtCesA2/3*, which group in two other clusters, are all expressed specifically in cells depositing secondary cell wall material (Turner and Somerville, 1997; Taylor et al., 2000; Wang and Loopstra, 1998; Wu et al., 2000). Based on expression studies and mutant analysis, the *CesA* genes from the other clusters are expected to be involved in primary cell wall cellulose synthesis. We therefore predict that the four potato *CesA* genes are also involved in primary cell wall cellulose synthesis.

### 2.3. Modification of CesA gene expression in potato plants

Several constructs were made in order to modulate cellulose biosynthesis during plant, and specifically tuber, development in potato. The full-length *StCesA3* cDNA sequence was cloned in both sense (SE) and antisense (AS) orientations in a pBIN19-derived vector. Expression was driven by the granule-bound starch synthase (gbss) promoter, which is highly active in potato tubers (Visser et al., 1991). The transformation efficiency of the sense and antisense constructs was 13 and 33% respectively (in comparison to 33% for an empty vector control). The generated SE- and AS-*CesA3* transformants developed normally and did not show any visual abnormalities (including the tubers) in comparison to wild-type plants. However, transformation with a construct in which antisense *StCesA3* was regulated by the constitutive CaMV 35S promoter (Benfey and Chua, 1990), gave a transformation efficiency of only 3% and was perhaps lethal. It is not clear if this construct was more efficient than the gbss constructs in obtaining extremely low cellulose levels, which subsequently resulted in plant death. It may also be caused by the difference in promoter activity, suggesting that the selection of an appropriate promoter is important for modulating cellulose levels in the plant.

Additonally, four constructs were made to modulate specifically the RNA expression levels of the four different potato *CesA* genes using only the CSR in antisense orientation under control of the gbss promoter. These constructs, and their corresponding transformants,

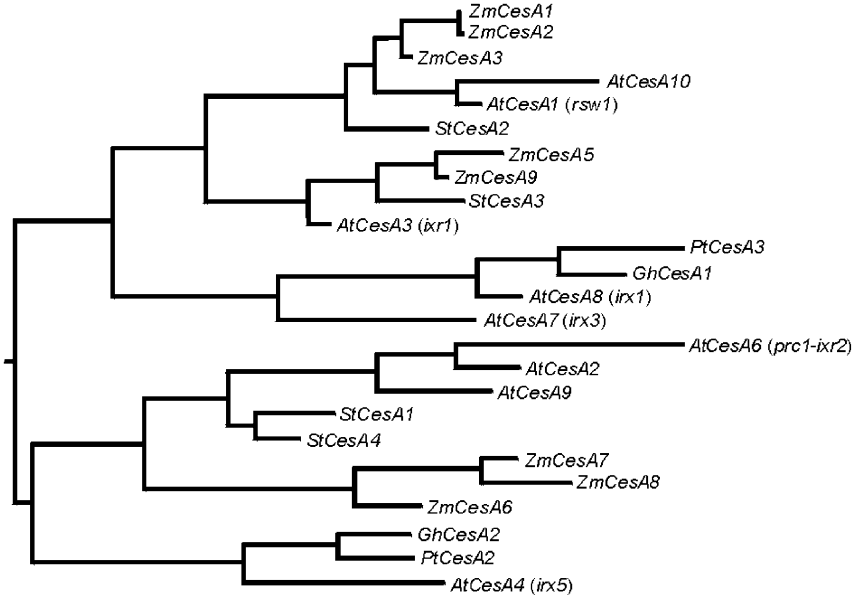


Fig. 1. Dendrogram representing the relationship between derived amino acid sequences of the class-specific regions (CSRs) among *CesA*s from Arabidopsis, cotton, poplar, maize and the four potato sequences. The following sequences were used: *Arabidopsis thaliana*; AF027172 (*AtCesA1*, *rsw1*), AF027173 (*AtCesA2*), AF027174 (*AtCesA3*, *ixr1*), BAB09063 (*AtCesA4, irx5*), AF062485 (*AtCesA6*, *prc1*/*ixr2*), AF088917 (*AtCesA7*, *irx3*), AL035526 (*AtCesA8*, *irx1*), aad20396 (*AtCesA9*), aad20713 (*AtCesA10*), *Gossypium hirsutum*; U58283 (*GhCesA1*), U58284 (*GhCesA2*), *Populus* tremuloides; AF081534 (*PtCesA2*), AF072131 (*PtCesA3*), Zea *mays*; AF200525 (*ZmCesA1*), AF200526 (*ZmCesA2*), AF200527 (*ZmCesA3*), AF200529 (*ZmCesA5*), AF200530 (*ZmCesA6*), AF200531 (*ZmCesA7*), AF200532 (*ZmCesA8*), AF200533 (*ZmCesA9*) *Solanum tuberosum;* AY221086 (*StCesA1*), AY221089 (*StCesA2*), AY221087 (*StCesA3*), AY221088 (*StCesA4*). The sequences were aligned using the Jotun Hein Method in the MegAlign program of the DNA-Star package with a gap penalty of 11, a gap-length penalty of 3, and a PAM250 weight table.

are referred to as csr1, 2, 3 and 4, where the numbers correspond with the *StCesA* clone. All constructs gave normal transformation efficiencies and the plants did not show any visible defects during growth and development in comparison with the WT plants. Tissue sections from tuber material were stained with Calcofluor White, which stains cellulose, and imaged by fluorescence microscopy, but no clear differences from sections of WT tuber were observed.

### 2.4. SE- and AS-CesA3 transformants show altered cellulose levels

Fourier Transform Infra Red microspectroscopy (FTIR) has been used as a rapid screen for transformants for alterations in their cell wall composition, as a preliminary to chemical analysis (Sørensen et al., 2000). Because both starch and cellulose give rise to absorbances in the carbohydrate fingerprint region of the IR spectrum (Chen et al., 1998) high starch content can potentially mask changes to cellulose content in the transformants. To circumvent this problem the tuber tissue sections were incubated for 15 min at 90 °C during which the starch gelatinises and is partially solubilised while the crystalline cellulose is not affected by this treatment.

Exploratory Principal Component Analysis (PCA) is used to reduce complexity in multivariate data, such as infrared spectra, to a few variables, termed principal components (PCs), that account for the variance in a set of observations (Kemsley, 1998). Each observation (spectrum) is given a score for each of the new variables and structure in the data set can be revealed by plotting the scores for one principal component against another. The PCs are ranked such that PC1 will account for most of the variance in the population and up to 10 PCs are calculated. Each PC is represented by a "loading", showing both positively and negatively correlated spectral features that are sometimes possible to interpret with structure-frequency correlation charts (Kemsley, 1998). In this case, we used exploratory PCA of infrared spectra collected from sections of transgenic tubers and tubers from control plants (50 transgenic lines which were transformed with an empty control vector) to indicate outliers from the control population (data not shown). From this preliminary selection, a large number of SE-*CesA3* transformants were identified and assigned as members of two groups (both discriminating differently from the control population), whilst the control plant spectra were assigned to a third group. Exploratory PCA was then carried out to see if there was any clustering within this new data set. Fig. 2a shows the cluster plot of the two groups of SE-*CesA3* transformants with control plants. The three groups separate in the cluster plot with some transformants having more negative scores and others having more positive scores with respect to the wild-type using PC3. PC loading 3 (Fig. 2b) shows characteristic features of pectin (Séné et

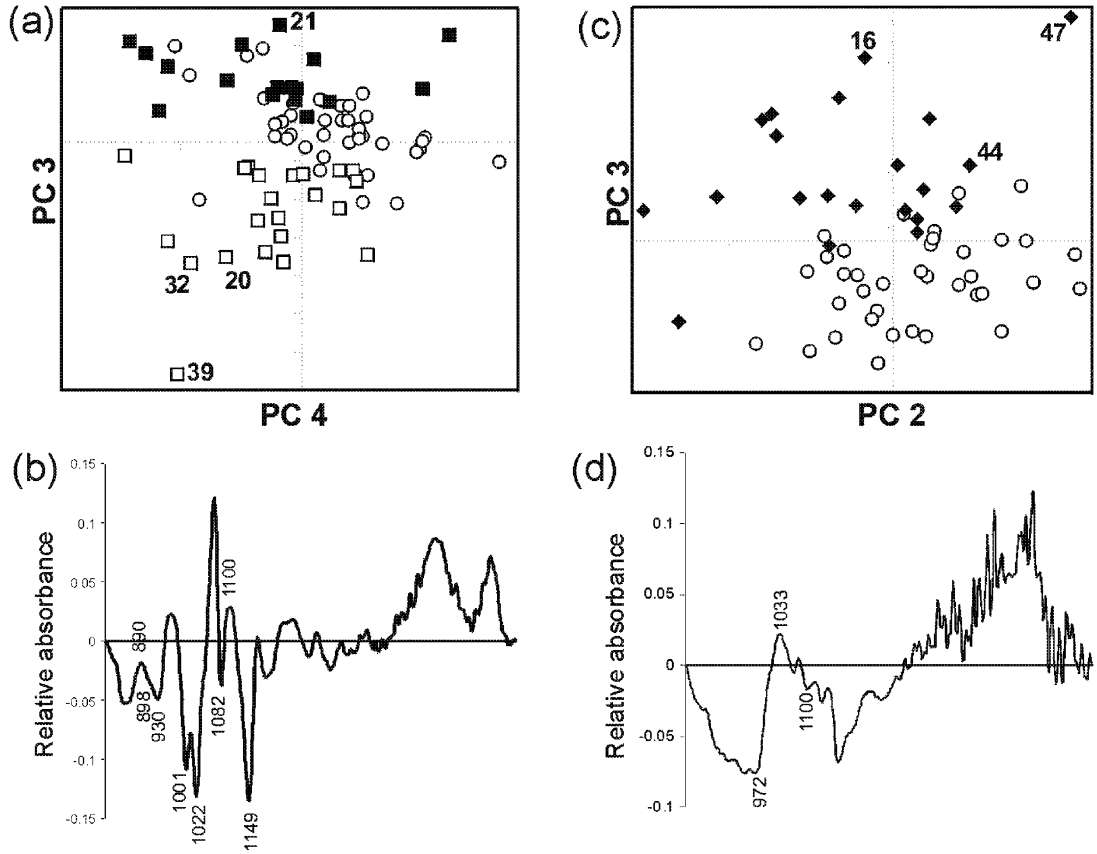


Fig. 2. Exploratory PCA of infrared spectra obtained from potato tubers transformed with StCesA3 constructs and WT tubers. (a) Exploratory PCA discriminates two groups of SE-*CesA* transformants (closed and open squares, respectively referring to plants discriminated from WT with either positive or negative PC scores) from the control plants (open circles) by using PC3; (b) PC loading 3 shows features characteristic of pectin (890, 1001, 1022, 1082 and 1149 $cm^{-1}$, Séné et al., 1994). SE-*CesA* transformants #20, 21, 32 and 39, were selected for further analysis; (c) Exploratory PCA discriminates the AS-*CesA* transformants (closed diamonds) from the control plants (open circles) by using PC3; (d) PC loading 3 shows features characteristic for cellulose (1033 $cm^{-1}$, Liang and Marchessault, 1959) and pectin (972 and 1100 $cm^{-1}$, Séné et al., 1994). AS-*CesA* transformants #16, 44 and 47 were selected for further analysis.

al., 1994; peaks at 890, 1001, 1022, 1082, and 1149 $cm^{-1}$). Unfortunately no signals were identified which could be assigned to differences in levels of cellulose. However, as changes in cell wall composition were indicated, 4 SE-*CesA3* transformants, #20, 21, 32 and 39, were selected for further analysis.

Similarly, AS-*CesA3* lines could be discriminated from the control-lines by PCA. Fig. 2c shows a cluster plot with group separation largely by PC3: as the calculation of PCs depends on the particular observations (spectra) in the data set, this PC3 differs from PC3 in Fig. 2a. Interpretation of this loading would be highly speculative, but the separation in the cluster plot indicates some changes in cell wall composition in these transgenic lines. Based on the cluster plot, the AS-*CesA3* transformants #47, 16, and 44, were selected for further analysis, predicting #47 and 16 to be most distant from the control plants.

For the selected transformants, tuber material from three tubers (of three individual plants) was pooled and used for two separate cell wall isolations. The cellulose content was determined by a colorimetric assay, and expressed as a percentage (w/w) of cellulose per total 'crude' cell wall preparation. As this 'crude' cell wall preparation consists of about 90% starch, cellulose levels are calculated at about 3–4% instead of the 30–40% expected in de-starched cell wall material (CWM, Ryden and Selvendran, 1990). This may also explain why a PC with characteristic features of cellulose was not obtained—random variation in starch content may account for most of the variance of individual observations. Fig. 3 shows the variation in cellulose content of a number of transformants and a WT control. The level of cellulose varies among the different transgenic plants some of which show a very low percentage (down to 2% for AS-*CesA3*-47 and SE-*CesA3*-21) of cellulose in comparison to the WT (4%). In the sense-expression lines, transformant SE-*CesA3*-39 (5 and 8% cellulose respectively) has an increased cellulose content in comparison to the WT, while SE-*CesA3*-21 and 32 show a decrease. Line 44 of the AS-*CesA3* plants has WT levels of cellulose while 47 and 16 have a reduced cellulose content.

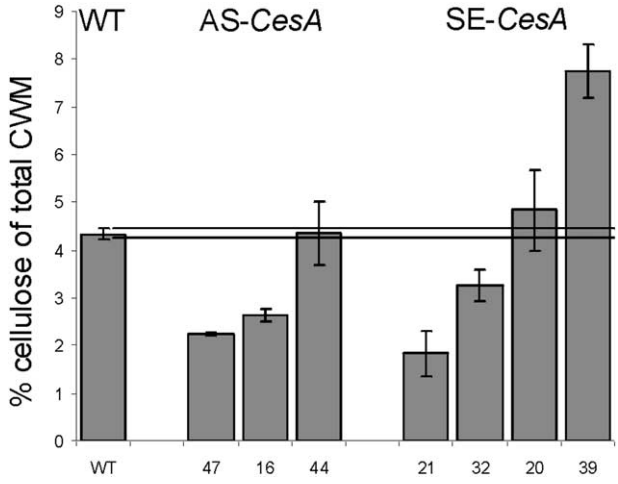


Fig. 3. Percentage of cellulose in a crude cell wall preparation from the transformed and control plants. The crude cell wall preparation was isolated from potato tubers and subsequently hydrolysed with TFA. The concentration of the remaining cellulose was determined by a colorimetric assay and subsequently expressed as percentage of the total crude cell wall preparation (including starch). Measurements were performed in duplicate and the bars represent the standard deviation.

We have performed several RT-PCR experiments (using RNA of the same tuber material) to study whether these modified cellulose contents are correlated to *StCesA3* expression levels. Different primer combinations were used to selectively determine the expression level of the four potato *CesA* genes and the *StUGE51* gene (UDP-Glc-4-epimerase from *Solanum tuberosum*, AY221085, Oomen et al., 2004), which is used as a control because of its constitutive expression. Fig. 4 shows the RT-PCR results for both the SE-*CesA3* and the AS-*CesA3* transformants with the *StCesA3* (a) and *StUGE51* (b) primers. Comparison of the amount of product amplified with the *StUGE51* primers and the *StCesA3* primers shows a variation of the *StCesA3* expression in the transformants in relation to the WT plant. *StCesA3* RNA expression is clearly lower in SE-*CesA3*-21 and higher in SE-*CesA3*-39. However, it is remarkable that the AS-*CesA3*-16 and 47 and SE-*CesA3*-32, which have reduced cellulose levels, show RNA expression levels similar to the WT plants.

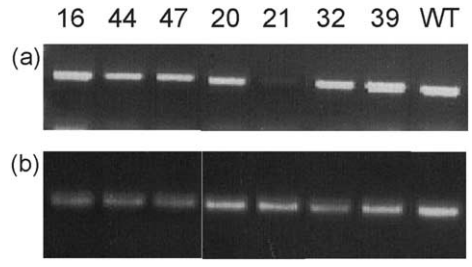


Fig. 4. RT-PCR analysis of SE- and AS-*CesA* transformants and wild type tuber RNA. Agarose gel of RT-PCR products amplified by using primers for *StCesA3* (a) and *StUGE51* (b).

### 2.5. Identification of StCesA CSR transformants with an altered cell wall composition using FTIR

The csr transformants were studied by FTIR and PCA to identify transgenic lines with altered wall composition that could be used for further analysis. The plants transformed with the csr3 construct could not be discriminated from the control plants by cluster plots of any pair of up to 10 PCs. Fig. 5a shows the PCA plot of PC loadings 1 and 2. We conclude that there is little or no difference between cell walls of WT and the csr3 lines.

For the other constructs (csr2, csr4, and csr1) the identification of transformants with a modified cell wall composition could be made. Fig. 5c shows discrimination of csr2 from the control plants mostly using PC3. A prediction from the cluster plot is that line 1 has a more extreme phenotype than line 18, whilst line 8 is close to the control plants cluster, and so these transformants were selected for further analysis. In this case, the corresponding loading for PC3 shows a peak (Fig. 5d) characteristic of cellulose at 1059 $cm^{-1}$ (Liang and Marchessault, 1959) and several pectin peaks (Séné et al., 1994; 1022, 1082 and 1144 $nm^{-1}$) indicating changes in these cell wall polymers. The loading indicates an increase in pectin content in the transformants while the negative cellulose peak suggests a decrease in cellulose. Exploratory PCA shows that the csr4 transformants separated from the control plants largely by PC3 (Fig. 5e). A similar loading is obtained with peaks inverted as the transformants now have negative scores for this PC: differences in pectin ($-1022$, $-1082$ and $-1144$ $nm^{-1}$) and cellulose ($+1059$ $nm^{-1}$) characteristics were found (Fig. 5f). The loading suggests an increase in pectin and a decrease in cellulose (positive peak) for most transformants. The transformants #11, 8 and 3, among which we predict the most extreme phenotype in line 11, were selected for further analysis.

Exploratory PCA indicates that the csr1 transformants can be discriminated from the control plants using a combination of PC 3 and 4 (Fig. 5g). Loading 4 (Fig. 5h) is very similar to both of the PC 3 loadings used to discriminate csr2 and csr4 from the control plants. Four csr1 transformants (#7, 21, 25 and 34) were selected, among which we predict that lines 7 and 25 will have more extreme phenotypes than lines 21 and 34.

### 2.6. CSR transformants show modifications in both cellulose and pectin content

Fig. 6 shows the cellulose content of the selected csr transformants and a WT control. The variation in cellulose levels is present both between constructs but also within the different transgenic lines harbouring the same construct. As expected from FTIR, the csr3 transformants showed a cellulose content comparable to WT

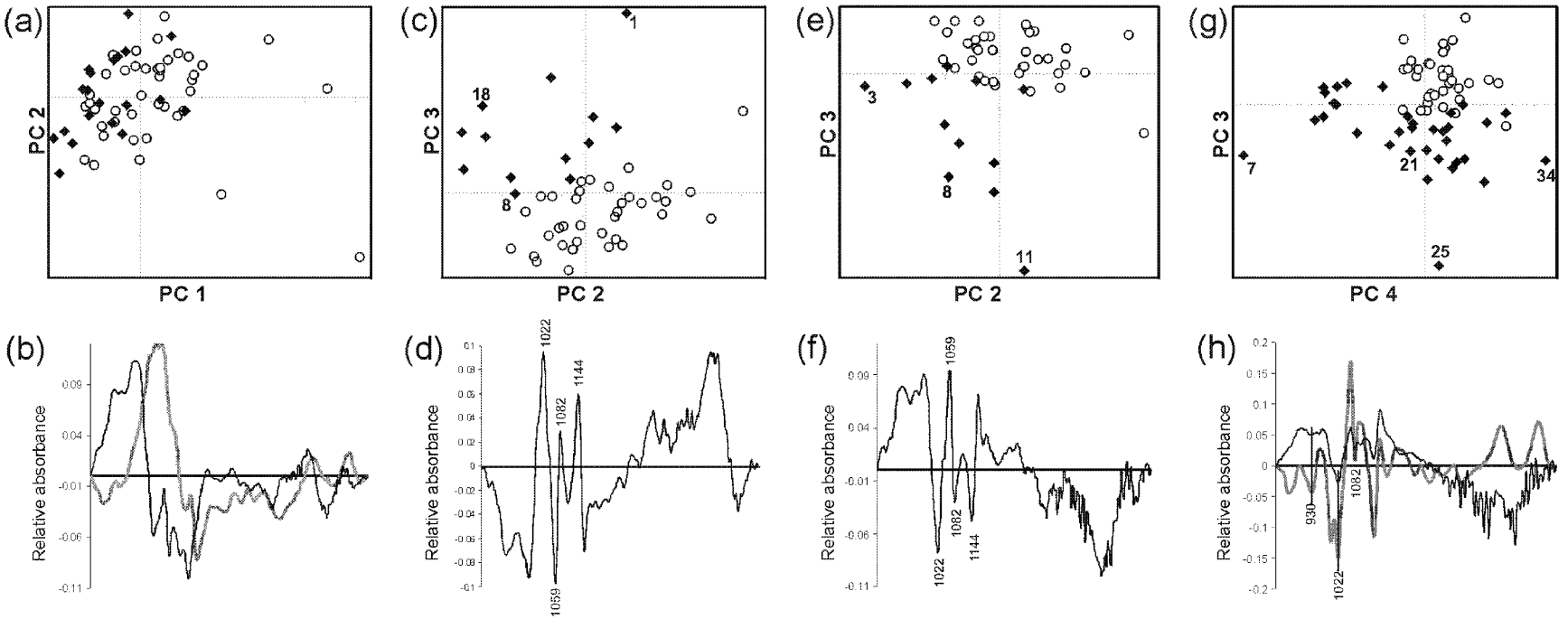


Fig. 5. FTIR and exploratory PCA of the potato csr transformants. Spectra from WT plants are represented by open circles and spectra from the respective transformants are represented by black diamonds. The corresponding PC loadings are shown on the × and y axis of a, c, e and f. Individual plants selected for further analysis are indicated by number. (a) *StCesA3* transformants and control plants can not be separated by PCA; (b) PC loadings 1 (grey line) and 2 (black line) do not show cellulose or pectin characteristics; (c) Exploratory PCA discriminates the *StCesA2* transformants and WT plants using 3 PC scores; (d) PC loading 3 shows characteristic pectin peaks (1022, 1082 and 1144 $nm^{-1}$) and a negative cellulose peak (1059 $nm^{-1}$); (e) The *StCesA4* transformants and WT plants are separated by exploratory PCA using 3 PC scores; (f) PC loading 3 shows features characteristic for both cellulose (1059 $nm^{-1}$) and pectin (1022, 1082 and 1144 $nm^{-1}$); (g) The *StCesA1* transformants are discriminated from the control plants by PC3 and PC4; (h) PC loadings 3 (black line) and 4 (grey line) showing negative peaks for pectin (1022 and 1082 $nm^{-1}$) and cellulose (930 $nm^{-1}$).

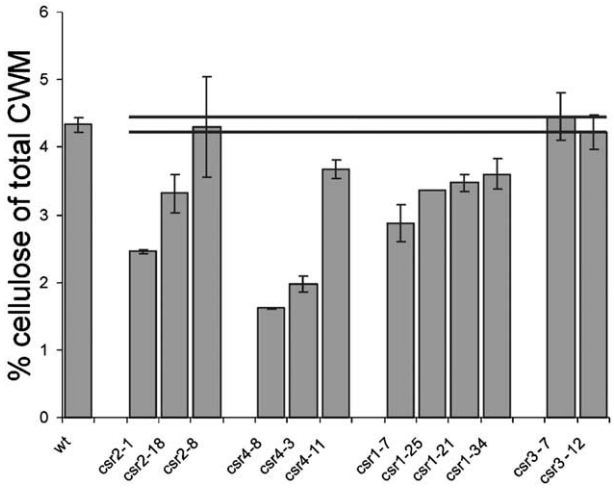


Fig. 6. Percentage of cellulose in a crude cell wall preparation from WT tubers and tubers transformed with csr constructs. Cellulose content of tuber material was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in a crude cell wall prepartion (including starch). All measurements were performed in duplicate and standard deviations are represented by the bars.

levels. The cellulose content of the csr2 transformants is clearly correlated to the PCA cluster plot showing transformant csr2-1 with the lowest cellulose content positioned furthest from the control plants. However, PC loadings reflect other changes in cell wall composition than cellulose content, so cellulose content alone is not the basis for the discrimination.

Transformant csr4-8, with a reduction of 60%, shows the lowest amount of cellulose identified in the selected plants. Based on the position of transformant csr4-11 in the PCA plot according to PC3, it was expected that this transformant was most distant from the control plants. However, transformants csr4-3 and 8 have much lower amounts of cellulose. The csr1 transformants show relatively small decreases in cellulose content (25% in comparison to 60% for csr4-8).

The monosaccharide compositions of the different transformants and a WT control is shown in Table 1. By de-starching isolated CWM, the amount of glucose was reduced to a level that allowed baseline separation of galactose, glucose and inositol with gas chromatography. To facilitate further comparison of the different monosaccharides, the glucose level was set to zero after which the relative mol% of the other monosaccharides was calculated (Table 1).

Comparison of the cellulose levels in the various transformants with the monosaccharide composition shows that, in the csr2 and 4 transformants, the lines with the lowest amount of cellulose have the most obvious changes in pectin composition (galactose and uronic acid content) and the lowest amounts of mannose. The csr1 transformants have mostly small changes in their pectin composition. However, overall there is no clear correlation between monosaccharide composition and cellulose content.

### 2.7. RT-PCR analysis of StCesA transcript levels in the CSR transformants

RT-PCR analysis indicates that the selected transformants show some modifications in their respective *StCesA* RNA expression levels. Among the three csr2 transformants (Fig. 7a), number 1 shows the most distinct decrease in *StCesA2* expression. For transformants 8 and 18 the decrease is less obvious, which fits the levels of cellulose found in these plants. In comparison to the WT control, the three csr4 transformants (Fig. 7b) neither show an obvious decrease in *StCesA4* expression, nor for any of the other *StCesA* genes. For the csr1 transformants (Fig. 7c) transformants number 7, 21 and 34 seem to show a decrease in *StCesA1* expression in comparison to the WT. However a similar variation

Table 1
Glycosyl residue composition (mol%) and cellulose content (% of crude cell wall preparation) isolated from tubers of wild type and *StCesA1, 2* and *4* CSR antisense expressing potato plants. The values represent the average of a duplicate analysis (two cell wall isolations of tuber material from three tubers of three individual plants) and include the standard deviation. To facilitate the comparison of the different sugars the Glc has been set zero because of the high starch content in potato tubers

| | Rha | Ara | Xyl | Man | Gal | UA | Cellulose |
|---|---|---|---|---|---|---|---|
| csr2-1 | 2±0.4 | 15±1.1 | 4±0.4 | 1±0.3 | 21±1.6 | 57±2.4 | 2.5±0.02 |
| csr2-18 | 3±0.6 | 12±0.0 | 6±0.2 | 2±0.3 | 36±1.2 | 41±1.1 | 3.3±0.29 |
| csr2-8 | 3±0.5 | 12±0.1 | 7±0.2 | 2±0.2 | 31±0.8 | 44±0.9 | 4.3±0.74 |
| csr4-8 | 3±0.3 | 11±0.5 | 5±0.4 | 0 | 33±1.0 | 47±0.1 | 1.6±0.01 |
| csr4-3 | 4±0.7 | 11±0.1 | 6±0.9 | 0 | 35±3.5 | 44±3.4 | 2.0±0.12 |
| csr4-11 | 3±0.3 | 13±0.4 | 7±0.4 | 2±0.3 | 39±0.4 | 36±1.2 | 3.7±0.13 |
| csr1-7 | 3±0.7 | 11±0.5 | 6±0.2 | 2±0.1 | 32±1.3 | 46±1.4 | 2.9±0.27 |
| csr1-25 | 5±1.1 | 11±0.2 | 7±1.3 | 2±0.2 | 31±1.8 | 44±4.2 | 3.4 |
| csr1-21 | 3±0.8 | 12±0.2 | 7±0.4 | 2±0.1 | 39±2.5 | 37±3.8 | 3.5±0.13 |
| csr1-34 | 3±0.2 | 12±0.8 | 6±1.0 | 6±5.2 | 33±0.9 | 41±4.2 | 3.6±0.22 |
| WT | 2±0.3 | 11±0.2 | 6±0.1 | 2±0.2 | 38±1.7 | 42±2.7 | 4.3±0.11 |

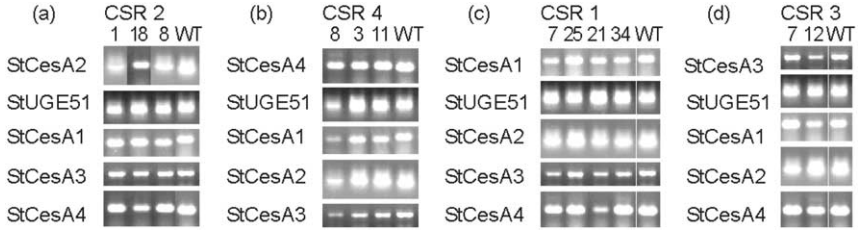


Fig. 7. RT-PCR analysis of CesA gene expression in tuber material derived of the selected transformants and wild type plants. Panels a, b, c and d show the PCR products visualised with EtBr after agarose gel electrophoresis for the csr2, csr4, csr1 and csr3 transformants, respectively. RT-PCR analyses were performed using the different specific *StCesA* primer pairs and a *StUGE51* primer pair which was used as a control because of its constitutive expression. In each panel the upper lane shows the amplification products using the *StCesA* primer pair corresponding to the CSR-construct introduced in these transformants. The lane below shows the control *StUGE51* amplification product while the other lanes show the results using the other *StCesA* primer pairs. Within each panel the transformants were ordered according to their increasing cellulose content from left to right.

in the expression pattern is observed with the *StCesA3* primers. Also, transformant csr1-21 shows less PCR product than the other plants in the csr1 series using the *StCesA4* primers. For the two csr3 transformants, number 12 shows a reduced expression both with the *StCesA3* as well as the *StCesA1* primer pair (Fig. 7d). Summarizing, we have not found consistent relationships between RNA transcript levels and cellulose content in the four transgenic CSR series.

## 3. Discussion

A transgenic approach was taken to establish the boundaries for up and down-regulation of cellulose content in potato tubers. One full-length and three partial sequences of *CesA* genes were identified in a cDNA library from swelling stolon tips. Transgenic plants were generated with sense and antisense expression of the full length *StCesA3* cDNA and also plants in which only the CSR region of the four potato *CesA* cDNAs was used. By using the gbss promoter, cellulose content was modulated in a specific organ, the potato tuber.

### 3.1. Four CesA genes were isolated from developing stolons

Based on the dendrogram representing the genetic relationship between the different plant *CesA* genes, we suggest that the different potato *CesA* clones may share functional similarity with e.g. the *Arabidopsis* genes in the three main clusters. Mutant and antisense plants with loss of the function of the *CesA1*, *CesA2*, *CesA3* and *CesA6* (Arioli et al., 1998; Burn et al., 2002; Desprez et al., 2002; Fagard et al., 2000) genes are all deficient in the biosynthesis of primary wall cellulose, whilst some genes not in these clusters are implicated in secondary wall cellulose biosynthesis (Burn et al., 2002; Taylor et al., 1999, 2000; Wang and Loopstra, 1998; Wu et al., 2000). As described by Vergara and Carpita (2001), these three clusters are likely to contain all primary cell wall cellulose synthases. This, together with the fact that all four potato cDNAs were isolated from a single cDNA-library generated from swelling stolon tips suggests that these potato *CesA* clones play a role in primary cell wall biosynthesis.

### 3.2. Down-regulation by constructs using only the class-specific region of CesA genes

The transformants expressing the csr1, 2 and 4 showed clear reductions in cellulose content, of which the csr4-8 transformant was the most pronounced one with a decrease down to 40% of the WT cellulose content. Additionally, FTIR showed to be very useful for the identification of these plants with a modified cellulose content from a larger group of transformants. Nevertheless, it can not be ignored that the reproducibly visualised transcript levels of the *StCesA* genes often do not correlate with the cellulose phenotype of the transformants (both for the csr as well as the full-length *StCesA3* plants) as shown by the cellulose determination and FTIR data. Since the analysis of the cellulose content and RT-PCR were performed on the same tuber material (a pooled fraction of tubers from 2 to 3 different plants of the same transgenic line) it is unlikely that the observed modifications are representing natural variation. Previous experiments in potato (Kuipers et al., 1994), studying the effects of antisense granule bound starch synthase expression on starch composition, already showed that the phenotypic effects (including protein levels) do not necessarily show a linear correlation to RNA expression levels of the corresponding genes. Similar discrepancies between transcript and protein levels were also found in yeast (Ideker et al., 2001).

In contrast to the other csr transformants the plants harbouring the csr3 construct do not show any modifications in their cell wall composition. This is remarkable, especially regarding the fact that the full-length

SE- and AS-*CesA3* plants do show a modulation of their cellulose content. It is of course possible that the modifications observed in the full-length sense and antisense transformants are a result of down-regulated expression of multiple potato *CesA* genes, rather than the endogenous *StCesA3* alone. However, as noted before, the RT-PCR results are not conclusive, which makes it difficult to substantiate a hypothesis of simultaneous inhibition of *CesA* gene expression. Additionally, this would require the identification of all other potato *CesA* genes, which have not been described in this study (for instance the *CesA* genes involved in secondary wall biosynthesis).

### 3.3. Up-regulation of cellulose content was obtained by over-expression of SE-CesA3

Several reports (Read and Bacic, 2002; Taylor et al., 2003) have focussed on the composition of the cellulose synthase complex and the importance of multiple *CesA* genes in one plant. It is still unclear how many *CesA* proteins can participate in one cellulose synthase complex, and whether all *CesA* subunits are equally important. The SE- and AS-*CesA3* transformants demonstrate that it is possible to down- (to 43%), but also up- (to 200%) regulate the cellulose content by modifying RNA expression levels. The increased level of cellulose in the SE-*CesA3*-39 transformant is remarkable and in contrast with the generally accepted idea that multiple different *CesA* proteins participate in one cellulose synthase complex (Desprez et al., 2002; Fagard et al., 2000; Taylor et al., 2003). This may imply that feedback mechanisms act to regulate gene expression of other CesA proteins in the cellulose synthase complex. Of course, this event has to be studied in more detail and the generation of more transformants over-expressing the *StCesA3* or other *CesA* genes will be necessary before further conclusions can be made.

The transgenic lines reported here have demonstrated the possibility to down-regulate the different potato *CesA* genes in potato tubers. These lines will be useful materials to reveal the specific function of these respective *CesA* genes. However, the isolation of all potato *CesA* genes and detailed functional analysis will be necessary to link a function to a particular *CesA* gene. Additionally, these plants showed some advantages of using such transformants in comparison with a mutant analysis approach. This was already indicated by the study from Burn et al. (2002) which showed the difficulty to extrapolate the results from antisense plants and plants harbouring a mutation in the same gene. In mutants, it is possible that the observed effects are caused by a modified activity/property of the corresponding enzyme. The antisense approach ensures that one is studying the effect of a decreased expression of the target gene. Further, with antisense, transgenic lines can be generated with a range of the phenotype of interest, particularly useful if a null mutation is potentially lethal.

## 4. Experimental

### 4.1. Amplification of the potato CesA fragment

Two primers were designed based on putative *CesA* sequences encoding the catalytic subunit of cellulose synthase in cotton, rice, and *Acetobacter xylinum* and subsequently adapted to the codon usage of potato. We used the following sequences; cotton (U58283 and U58284), rice (D47622 and D41986), *Acetobacter* Bcs_A (M37202), *Acetobacter* Acs_A (X54676) and *Acetobacter* Acs-AII (U15957) as described in Pear et al., 1996; Saxena et al., 1990; Saxena and Brown, 1995; Wong et al., 1990. The primers are located in the U1 and U2 regions (which are highly conserved) of the *CesA* genes to amplify the Plant-Conserved Region. The sequence of the two primers is: CELS1, 5′-cccagttgataaggtttcatgcta-3′; CELS2, 5′-attaaggatgaagggagcatttgt-3′.

PCR amplification was performed with cDNA originating from young potato leaves using 50 ng of both CELS primers, standard Perkin Elmer PCR buffer with $MgCl_2$ added to a final concentration of 3.5 mM, and 1.25 units of *Taq* polymerase (Perkin Elmer). Thirty-five cycles of PCR were performed using a cycle profile of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and amplification at 72 °C for 30 s. The amplification product was cloned into the pGEM-T Easy cloning vector (Promega) according to the instructions of the manufacturer. The clones were sequenced with the pUC/M13 Forward and pUC/M13 Reverse primers.

### 4.2. Screening of the cDNA library

The PCR fragment, representing the Plant-Conserved Region (P-CR) region of the *CesA* gene, was used to screen a λZAPII cDNA library (Stratagene) that was made from poly-$A^+$ RNA isolated from swelling stolon tips (kindly supplied by Dr. M. Taylor SCRI, Scotland). Approximately $10^6$ plaques from the λ-phage cDNA library were screened using the amplification product of the PCR labelled with the rediprime II random prime labelling kit (Amersham pharmacia biotech). Hybridizations were done in modified Church buffer (Church and Gilbert, 1984) for 12 h at 60 °C (low stringency to enable the isolation of multiple *CesA* cDNA clones) followed by washes in 2×, 1× and 0.5× SSPE, 0.1% SDS at 60 °C, each 2 × 15 min.

The screening yielded 11 positive clones, of which the phagemids were excised using the ExAssist Interference-Resistent Helper Phage (Stratagene), resulting in a pBluescript vector containing the cDNA. The clones were sequenced using the T3 (5′-aattaaccctcactaaaggg-3′)

and T7 (5′-gtaatacgactcactatagggc-3′) primer and cDNA sequence-specific primers.

### 4.3. Sequence analysis

Sequencing reactions were performed using an automated ABI-sequencer (ABI-Perkin Elmer B.V. Oosterhout NL). The cDNA sequences were analyzed for homology with known sequences in the databases using the BLAST programs. For further analysis and comparison of all the sequences we used Clustal analysis and One pair alignment of the DNA-Star program package. Four different potato *CesA* clones were identified and entered into the public database; AY221086 (*StCesA1*), AY221089 (*StCesA2*), AY221087 (*StCesA3*) and AY221088 (*StCesA4*).

### 4.4. Vector construction and transformation of potato plants

The full length *StCesA3* clone was used to make both sense and antisense constructs with the granule-bound starch synthase (GBSS) promoter (Visser et al., 1991) and the CaMV 35S promoter (Benfey and Chua, 1990). The gbss promoter was isolated from the pPGB-1S vector (Kuipers et al., 1995) by a *HindIII*/*BamHI* digestion and ligated into a pBluescript vector. Subsequently, this fragment was isolated by a *HindIII*/*SpeI* digestion and ligated into the pBIN20 vector (Hennegan and Danna, 1998) generating the pBIN20gbss. The *StCesA*3 cDNA was bi-directionally cloned as an *XbaI*/*XhoI* fragment into the pBIN20gbss, containing two *XbaI* sites with a *XhoI* site in between. The two sense and antisense constructs with the CaMV 35S promoter were made in the same fashion as the gbss promoter constructs after *HindIII*/*BamHI* digestion of the pBI121s (Jefferson et al., 1987) vector to obtain the CaMV 35S promoter.

Antisense constructs containing only the CSR of the four potato *CesA* clones were generated using the pBIN20gbss vector. The CSR was PCR amplified with specific primers; *StCesA*2; Forward 5′-caggcaggctctatacgggt-3′, Reverse 5′-ccattcagtcttgtcctcgta-3′, *StCesA*4; Forward 5′-gaggcaagcgctttatggatat-3′, Reverse 5′-ccccattcagttttgtcttcata-3′, *StCesA*1; Forward 5′-gaggcaagcccttatggttat-3′, Reverse 5′-ccccattctgttttgtcttcata-3′, *StCesA*3; Forward 5′-cagctttatatggttatgaacct-3′, Reverse 5′-ccccattctgatttatcttcata-3′. The amplified fragment was ligated in the pGEM-T Easy cloning vector (Promega, Leiden, The Netherlands), excised with *EcoRI* and ligated into the *EcoRI* cleaved pBIN20gbss vector. All constructs were checked for sense or antisense orientation of the inserted cDNA by PCR amplification using cDNA and promoter specific primers.

In vitro shoots of the *Solanum tuberosum* cultivar Kardal were used for *Agrobacterium tumefaciens*-mediated transformation (Visser et al., 1989). After regeneration of in vitro shoots on selective kanamycin medium, 30–50 shoots per construct were transferred to the greenhouse to generate mature plants.

### 4.5. Fourier transform infra red microspectroscopy

Vibratome sections (70 μm) from all freshly harvested wild-type and transgenic tubers (one section from one tuber per transgenic line or WT plant) were heated in water at 90 °C for 15 min (to gelatinize the starch) and subsequently mounted on barium fluoride windows and air-dried. The barium fluoride window was supported on the stage of a UMA500 microscope accessory of a Bio-Rad FTS175c FTIR spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. For each section, three areas of 100 $\mu m^2$ of the cortex region were selected and spectra obtained. Sixty-four interferograms were collected in transmission mode with 8 $cm^{-1}$ resolution and were co-added to improve the signal-to-noise ratio for each sample. The three spectra per section were averaged and subsequently baseline-corrected and area-normalized. Exploratory Principal Component Analysis (PCA) of area-normalized spectra in the region 1800–800 $cm^{-1}$ was carried out by using WIN-DISCRIM software (E.K. Kemsley, Institute of Food Research, Norwich, U.K.).

### 4.6. RT-PCR method

Tubers of two or three transformed plants (one from each) were pooled and ground to a fine powder in liquid nitrogen. RNA was extracted as described by Kuipers et al. (1994). Fifty micrograms of total RNA was treated with DNAseI and purified using the GenElute™ Mammalian Total RNA Kit (Sigma, Zwijndrecht, The Netherlands). Nine point five micrograms of RNA was used for cDNA synthesis in a mix containing, 100 ng primer polydT (5′-ttttttttttttttttttttttttt-3′), 1 μl 25 mM dNTPs, 2 μl 100 mM DTT, 10 μl 5× First Strand Buffer and 1 μl Reverse transcriptase/ Superscript™ II (Life Technologies, Breda, The Netherlands) in a final volume of 50 μl for 1 h at 42 °C. Five microlitres of cDNA was used in a standard PCR reaction. Several experiments were performed to optimize cycle number and Tm (for each primer pair), in such a way that easily detectable, but submaximal amounts of DNA were amplified. Based on these experiments the following primer/Tm/cycle-number combinations were used. *StUGE51* primers, F(5′-ttggtaaccccaaggatttt-3′), R(5′-tttagcggacggtcatgttgttgc-3′) Tm = 55 °C, 28 cycles. *StCesA3* primers, F(5′-gatggaaatgagttgccacgacta-3′), R(5′-ccacttcaaccgtccattgtatcc-3′) Tm = 58.5 °C, 22 cycles. *StCesA1* primers, F(5′-ggccgaaatggtgttgctgttgt-3′), R(5′-cctatcgggcatgcagtagacg-3′) Tm = 67 °C, 23 cycles. *StCesA2* primers, F(5′-gtgttgttttaacaggcaggctctatac-3′),

R(5′-aggcttagtggatgcgggaatg-3′) Tm = 58.3 °C, 23 cycles. *StCesA4* primers, F(5′-ttctttttatcggtggtcgttttcc-3′), R(5′-tgcgggtgtcagcggtcatc-3′) Tm = 51 °C, 31 cycles.

### 4.7. Isolation of cell wall material (CWM) from potato tubers

For the isolation of CWM, three tubers (of three individual plants from the same transgenic line) 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 RT with continuous shaking. The CWM was pelleted by centrifugation at 16 000 rpm for 14 min. Subsequently, the residue was washed with water, ethanol, acetone and air-dried. For determination of the neutral sugar composition, 300 mg of CWM was resuspended in water, heated for 10 min at 95 °C to gelatinize the starch, followed by incubation with α-amylase (Boehringer, Germany) and pullulanase (Megazyme, Ireland) to degrade the starch. After incubation, the CWM was precipitated in 70% ethanol, by adding an appropriate volume of 96% ethanol to the reaction mixture, and washed with acetone.

### 4.8. Analysis of the cellulose content

Fifty milligrams of CWM was incubated for 90 min at 120 °C in 5 ml 2M TFA. The remaining cellulose was pelleted and washed with water and ethanol. The pellet was solubilized in 67% (v/v) $H_2SO_4$ and diluted appropriately to determine the cellulose content colorimetrically using anthrone as a coloring agent according to Updegraff (1969). This analysis was performed in duplicate and standard deviations were calculated accordingly.

### 4.9. Analysis of the neutral sugars and uronic acid content

For de-starched CWM, the neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples were pre-treated with 72% (w/w) $H_2SO_4$ (1 h, 30 °C) followed by hydrolysis with 1M $H_2SO_4$ for 3 h at 100 °C and the constituent sugars were analyzed as their alditol acetates by gas chromatography with FID. The same hydrolysate was diluted and used for the determination of the uronic acid content. Concentrated $H_2SO_4$, containing 0.0125M $Na_2B_4O_7$, was used in an automated *m*-hydroxydiphenyl test (Thibault and Robin, 1975) to determine the uronic acid content colorimetrically. Galacturonic acid was used as a standard. This analysis was performed in duplo and standard deviations were calculated accordingly.

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