Indian Journal of Science and Technology Vol.2 No 3 (Mar. 2009) ISSN: 0974- 6846

Irregular deposition of cell wall polymers resulting from defective cellulose synthase complexes

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Abstract: The crystalline cellulose microfibril is formed by the spontaneous association of about 36 β-D-glucan chains, which are simultaneously synthesised by a large membrane-localised multi-enzyme cellulose synthase complex. Antisense technology has been previously employed separately on two of the constituent cellulose synthase catalytic subunits (CesA) of the complex in potato (*Solanum tuberosum*), namely CesA2 and CesA4, to generate potato tuber cell walls with reduced cellulose content. Genetic crossing of two transgenic potato lines csr2-1 and csr4-8 was carried out to investigate the effects of two defective CesAs in the same genetic background, with respect to cellulose deposition in the potato tuber cell walls. It was striking to observe, through fluorescence microscopy with calcofluor white, a strong fluorescence in the cell corners and less prominent and uneven fluorescence around the cells of the csr2 tubers as compared to others. It was also noted that these phenotypes were not pronounced in the csr2/csr4 double transformants as expected.

Keywords: Cell wall, cellulose synthase, polysaccharide deposition,*Solanum tuberosum.*

Introduction

The crystalline cellulose microfibril is generally believed to be formed by the spontaneous association of about 36 β-D-glucan chains, which are simultaneously synthesised by a large membrane-localised multi-enzyme cellulose synthase complex that was revealed by microscopy (Tsekos & Reiss, 1992). Existing evidences indicate that the cellulose synthase complexes that assemble primary and secondary cell walls are each composed of three distinct CesA catalytic subunits (Fagard *et al*., 2000; Scheible *et al*., 2001; Desprez *et al*., 2002; Robert *et al*., 2004).

With the experimental evidence that CesA proteins can form homodimers through their N-terminal ring-finger domain (Kurek *et al*., 2002), it was suggested each complex subunit would contain three homodimers of distinct CesA isoforms. It is therefore not surprising to observe that impairment of a CesA isoform in the *rsw1* (radial swelling) mutant of *Arabidopsis thaliana* AtCesA1 (Arioli *et al*., 1998), affects the normal assembly of the CesA complex and consequently, cellulose deposition. Impairment of the CesA complex also alters cellulose synthesis and deposition in two other primary cell wall mutants *prc1* (procuste, AtCesA6) and *eli1* (ectopic lignification, AtCesA3) (Arioli *et al*., 1998; Cano-Delgado *et al*., 2000).

Additionally, it has been proposed that complex interactions exist among metabolic pathways leading to cellulose biosynthesis in plants (Joshi & Mansfield, 2007), such that mutation or down regulation of a particular gene can affect an associated metabolic process (Zenoni *et al*., 2004; Howles *et al*., 2006). As such, impairment to the complexes would not only have severe implication on cellulose deposition but also on the processes they influence.

Oomen *et al*. (2004) reported varying degrees of up- and down-regulation of cellulose content in the potato tuber cell walls. These transgenic potato tuber clones were obtained by transforming the potato plant with antisense/sense constructs of a complete cDNA of potato cellulose synthase, (AS-CesA3/SE-CesA3) and antisense constructs of the class-specific regions (CSR) of four corresponding potato cellulose synthase genes (CSR1, 2, 3 and 4). Two transgenic lines csr2-1 and csr4-8, which showed considerable cellulose reduction (40% and 60%, respectively) in their tuber cell walls were identified by the anthrone colorimetric assay (Oomen *et al*., 2004). These two lines also grouped together in the same cluster following Fourier Transform Infra Red Spectroscopy and Principal Component Analysis. These observations led to the speculation that CesA2 and CesA4 proteins are probably part of the same complex. Hence the interest to explore the possibility of achieving double transformants with combined effects of the two transgenes on cellulose deposition.

In this study, genetic crossing was made between the transgenic potato lines csr2-1 and csr4-8, whose tuber cell walls exhibit low levels of cellulose as compared to the wild type control. The offspring was screened and microscopically analysed for tuber cell wall phenotypes. The paper reports remarkable cell wall phenotypes in the offspring plants containing the csr2 construct and proffers possible explanations for the phenomenon.

Materials and methods

Plant material and growth conditions

Potato (*Solanum tuberosum*) plants used for the cross carried, in antisense orientation, csr2 sequence of the potato CesA2 gene (accession number AY221089) and the csr4 sequence of the CesA4 gene (accession number AY221088) as earlier reported (Obembe *et al*., 2008).

Light microscopy

One mm-thick potato tuber sections were fixed, dehydrated and embedded in Technovit 7100 resin (Kuroiwa *et al*. 1990) as earlier described by Obembe and Vincken (2008). 4 micron-thick tissue sections were stained with calcofluorwhite (0.04%) and examined by light microscope. Each microscopic examination was done in triplicates (tuber sections of three individual plants from each clone). To verify the observation made on the calcofluorwhite-stained csr2 tuber sections, fresh staining was made for csr2 and the control tuber sections

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with 0.1 % aniline blue in 0.1 M K_3PO_4 and examined under fluorescence microscope. This was then followed by calcofluorwhite staining of the aniline blue-stained tuber sections. They were then examined under fluorescence microscope for differential staining patterns.

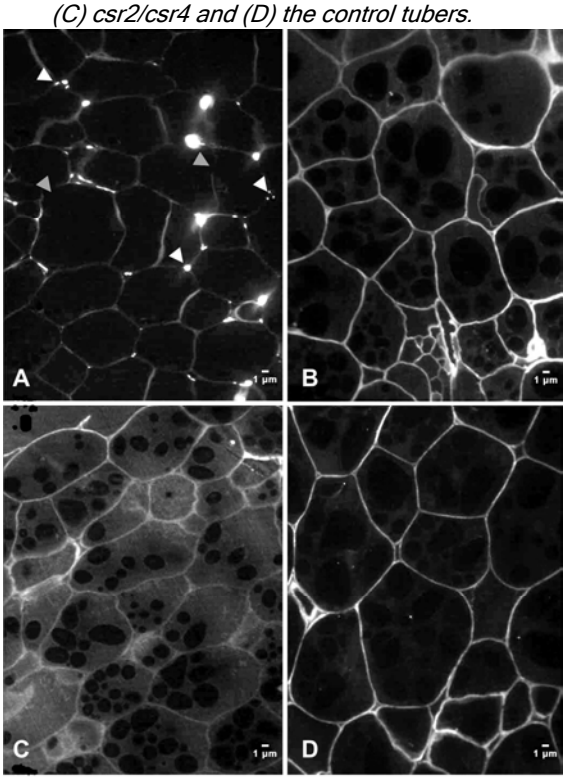
BMM-embedding and xyloglucanase treatment

In order to perform enzyme treatment on stem section, a different method of fixation and embedding in comparison with the one described before was necessary. Tuber samples were fixed in 5% glutardialdehyde in 0.1 M phosphate buffer, containing 0.1% Triton x100 for 2 hours. The samples were then washed with the buffer without glutardialdehyde, and dehydrated in an ethanol series (0, 10, 30, 50% ethanol containing 10 mM dithiothreitol (DDT)) and embedded in butyl-methyl methacrylat (BMM) as described elsewhere (Baskin *et al*., 1992; Gubler, 1989). 4 micron-thick tissue sections were made from the BMM-embedded stem samples and mounted on glass slides. The embedding material was removed from the sections with acetone, and then the sections were immediately washed with 25 mM citrate buffer pH 3.5. Incubation of glass-mounted sections was done at room temperature in 20 ml 25 mM citrate buffer pH 3.5 containing 5 U/mL of an endo-beta-1,4-glucanase from *Aspergillus aculeatus*, which is specific for xyloglucan (Pauly *et al*., 1999). It can be calculated that a stem section of approximately 1 cm^2 area and 4 µm in thickness contains approximately 10^{-2} µmol of linkages (as part of xyloglucan) that can be cleaved. Further assumptions were that 5% of the section consists of cell wall material, 20% of which is xyloglucan, and that the average molecular mass of a xyloglucan oligosaccharide is about 1000 Da. In order to ensure that pectin did not hinder the access of the xyloglucanase to xyloglucan, 1 U/mL pectin methyl esterase (PME) from *Aspergillus niger* and 5 U/mL polygalacturonase II (PG II) from *A. aculeatus* were added to the incubation mixture. After treatment, the sections were stained and observed under the microscope, and the incubation buffers were subjected to (MALDI TOF MS) and HPAEC analyses, to check for the release of xyloglucan oligosaccharides. In parallel, 15 mg of potato xyloglucan (extracted with 6 M KOH) was incubated in 1.5 mL 25mM citrate buffer pH 3.5 containing 20 U/mL of an endo-beta-1,4-glucanase, as the control sample. The incubation of the control and the sections were performed for 24 h at room temperature.

Results and discussion

Fluorescence microscopy with calcofluor white staining revealed an unusually strong fluorescence in cell wall corners of the csr2 tubers (Fig. 1A). This irregular deposition of wall materials was observed in all tissues except for the epidermis. Additionally, the fluorescence in the cell walls of csr2 tubers is much less prominent and mostly uneven as compared to the control, the csr4 and the double csr2/csr4 transformants. This strong fluorescence in cell wall corners of the csr2 potato tuber is indicative of preferential deposition of cellulosic polymers, although one cannot exclude the possibility that it might be xyloglucan. Hence, it was imperative to verify that the observed fluorescence in cell corners was actually from cellulose deposit since calcofluor white stains both cellulose and other β-glucan chain polysaccharides, such as callose and xyloglucan (Wood, 1980). In order to discriminate between cellulose and callose deposit, a step-wise staining, with aniline blue and calcofluor white was made, which indicated that the deposit was not callose (Fig. 2). This confirmation was based on the observation that aniline blue, which stains callose specifically (Yim & Bradford, 1998), did not give fluorescence in the cell wall corners. Selective degradation of xyloglucan by a xyloglucan-specific endoglucanase (xyloglucanase) (Pauly *et al*., 1999) was used for discriminating between cellulose and xyloglucan. PME and PG II were included in the enzyme mixture to ensure that pectin did not prevent the access of the xyloglucanase to xyloglucan. After the enzyme treatment, light micrographs of stained sections were similar to Figures 1A and 2B, which indicates that either the deposit is not xyloglucan, or that the enzyme treatment was not effective. Appropriate precautions were taken to ensure that embedding and de-waxing would not influence enzyme activity; similar procedures were used by others for antibody labelling of sections (Baskin *et al*., 1992; Gubler, 1989). It was verified that the xyloglucanase was active by incubating a potato xyloglucan with the enzyme. The release of the xyloglucan oligosaccharides was

Fig.1. Calcofluor white staining of technovit-embedded tuber sections. Fluorescence micrographs of perimedullary tissues of (A) csr2, (B) csr4, (C) csr2/csr4 and (D) the control tubers.



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shown with high-pH anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS) (data not shown), indicating that enzyme was active. However, it had been difficult to detect xyloglucan oligosaccharides released from the sections into the incubation buffer upon xyloglucanase treatment, even after 100x concentration of the incubation buffer by SepPak C_{18} solid-phase extraction. For incubation of the section, a higher xyloglucanase-to-substrate ratio was used than for the potato xyloglucan, which indicates that the amount of the enzyme added to the section should have been sufficient. It is possible that the amount of oligosaccharides released from the section is simply too low to be detected. Thus, the experiments hint that the corner deposit is cellulose, but the possibility of xyloglucan can not be excluded.

In the event that the corner deposit is cellulose, the following explanations can be put forward. Due to antisense suppression of CesA2, a principal CesA protein in the potato tuber (unpublished data), it is plausible that not enough of it is available for interaction with the other protein partners in the cellulose synthase complex. Based on the evidence of homodimerisation of CesA proteins (Kurek *et al*., 2002), it is expected that the other two CesA proteins will interact and might assemble a defective complex of 24 subunits. The implication of this would thus be a mixture of normal and defective complexes in the csr2 tubers since a complete knockout of the CesA2 gene was not observed (unpublished data). One consequence of the defective complex could be the synthesis of cellulose microfibrils having less glucan chains, being 24 instead of 36, which would presumably be weaker and more flexible. Based on the theory of ordering of cellulose microfibrils (Emons & Mulder, 1998), which proposes that the movement of cellulose synthase complex is driven by the forces generated by the polymerisation and crystallisation of the cellulose chains and, which has recently been reinforced by the biophysical model for the propulsion of the cellulose synthase complex (Diotallevi & Mulder, 2007), it is conceivable that cellulose deposition by the defective complexes would be abnormal. Preferential cellulose deposition in cell wall corners was also observed in cellulose-deficient *Arabidopsis* primary cell wall mutants *rsw1* (AtCesA1), *eli1* (AtCesA3) and *kor1* (Nicol *et al*., 1998; Cano-Delgado *et al*., 2000). It is intriguing that the cellulose reduction in the double csr2/csr4 transformant tuber cell walls does not show the phenotypes displayed by the csr2 cell walls. The observed even deposition of cellulose in the double csr2/csr4 transformant tuber cell walls might be explained by the biosynthetic activity of remnant but intact complexes. These complexes would produce normal but less cellulose microfibrils, which are evenly deposited round the cell wall.

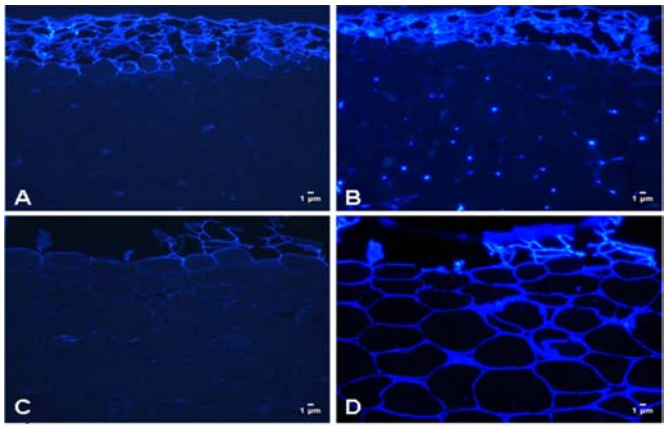
Alternatively, if the deposit is xyloglucan, it might be explained as follows. Due to a reduced amount of cellulose, xyloglucan cannot be kept in the wall, and diffuses to the cell corners. It has been observed before (Oomen *et al*., 2002), that galactan can move to the cell corner of potato tissue when it is released from rhamnogalacturonan by rhamnogalacturonan lyase.

On the whole, these results attest to the fact that impairment to a primary cell wall complex could have significant influence on cellulose deposition. They have also shed more light to the dynamics of cellulose deposition. However, more work still needs to be done to unravel the exact underlying mechanisms of rosette assembly and the precise nature of cellulose deposition.

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. I would like to thank Hauke Hilz from the Laboratory of Food Chemistry (Wageningen University) for performing HPAEC and MS analyses for xyloglucan oligosaccharides.

Fig.2. Double staining with aniline blue and calcofluor white to confirm that wall corner deposit is not callose. Micrographs (A) and (B) are csr2 embedded sections stained with aniline blue and calcofluor, respectively. Micrographs (C) and (D) are control embedded sections stained with aniline blue and calcofluor, respectively.



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