In planta modification of the potato tuber cell wall

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General introduction[1]

INTRODUCTION

After introduction of the cultivated potato (*Solanum tuberosum* L.) from the Andes in South America to Europe in the 16th century (Hawkes, 1978), the potato has spread throughout the world. It is currently, globally, the fourth most important food crop after wheat, rice and maize (http://apps.fao.org). Apart from its many uses for consumption (boiled, or processed as e.g. French fries and crisps), about one third of the potatoes grown in the Netherlands concerns starch potatoes. From these potatoes, in which starch accounts for 80% of the tuber's dry weight material, starch is obtained by industrial processing. The potato starch is used in several food and non-food applications as e.g. textile, paper, chemical and pharmaceutical industry. Cell wall fibres comprise a large portion of the waste material remaining after the starch isolation process. In contrast to some other plants (e.g. citrus fruits) the potato cell wall material is not used in any high value food or non-food applications and is currently only used in cattle feed. It has been suggested that after a specific modification of some potato cell wall polysaccharides these may be used in industrial applications as well (Vincken *et al.*, 1999).

POTATO TUBER CELL WALL COMPOSITION

The plant cell wall has been studied for many purposes and has shown to be a complex composite of many polysaccharides and proteins whose exact composition depends on the cell type (Carpita and Gibeau, 1993; Fry, 1995). By providing strength to the cells, their walls enable them to withstand turgor pressure and additionally determine size and shape of the cells. By providing rigidity to the plant cells (in comparison to animal systems) the cell walls function as the skeleton of the plant. The different cell wall polysaccharides are believed to have their specific function in determining wall characteristics. Figure 1 shows the cell wall polysaccharides and ratio in which they are present in potato tubers (Jarvis *et al.*, 1981; Vincken *et al.*, 1999).

FIGURE 1.
Polysaccharide composition (% w/w) of the potato cell wall, representing all pectic polysaccharides, (except RG II) in black (56%), and other cell wall polysaccharides in gray (Vincken, et al., 1999).

mannan (3%)
homogalacturonan (14%)
cellulose (30%)
rhamnogalacturonan I (6%)
galactan (28%)
xyloglucan (11%)
arabinan (8%)
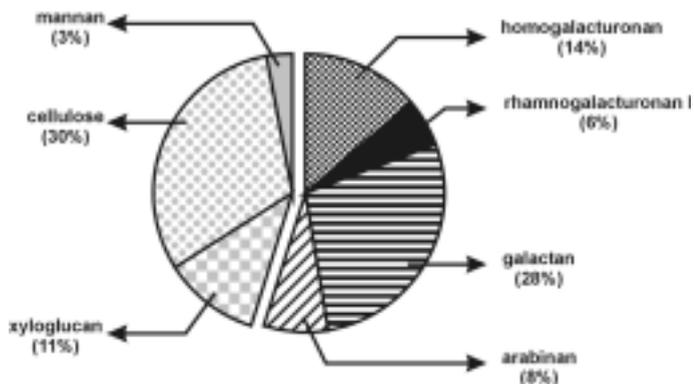
The homogalacturonan (HGA), rhamnogalacturonan (RG), galactan and arabinan are all part of the so-called pectic polysaccharides. Obviously, pectin is one of the major components of the potato cell wall, comprising about 56% of the total cell wall material. These pectic polysaccharides are suggested to form a matrix in the primary cell wall by Ca^{2+} cross linking of homogalcturonan stretches and boron cross linking of rhamnogalacturonan II. A second network in the primary wall is the cellulose/xyloglucan network. Both networks are believed to act independently in the cell wall as described by McCann and Roberts (1991; Figure 2). The pectin network is suggested to structurally regulate the pore-size of the wall. The cellulose/xyloglucan network, which is embedded in the pectin matrix, is believed to be the load-bearing network (Carpita and Gibeaut, 1993; McCann and Roberts, 1991).
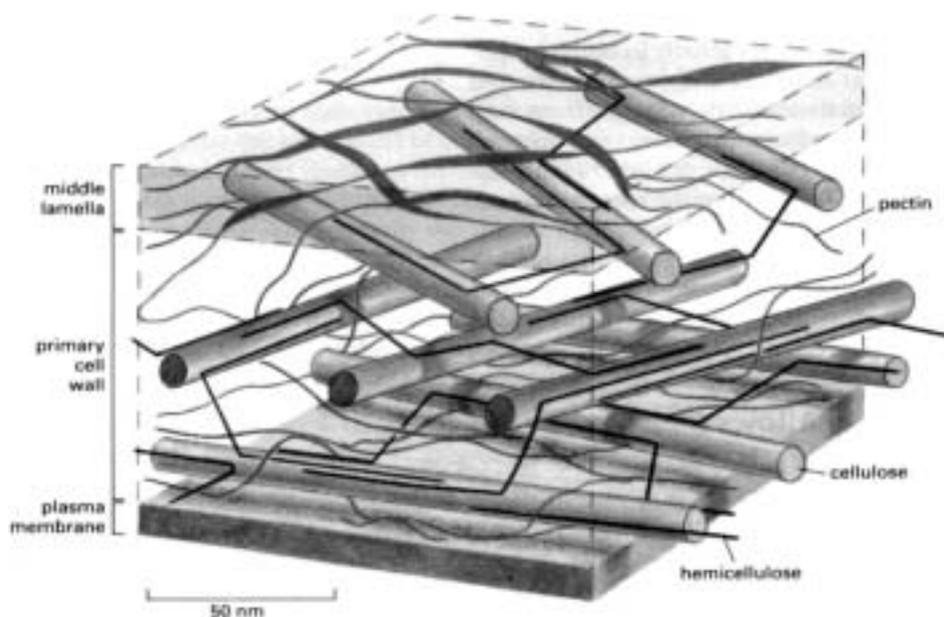
FIGURE 2.
A model for the cell wall of onion parenchyma tissue (McCann and Roberts, 1991). Scale bar represents 50 nm.

The primary structure of the individual pectic polysaccharides is well established. They comprise mainly homogalacturonan (HGA) and rhamnogalacturonan I (RG I). HGA (the smooth region) is composed of unbranched α-1→4-linked galacturonic acid (GalA) residues, which may be methyl-esterified and/or *O*-acetylated (Carpita and Gibeaut, 1993). RG I has a backbone composed of repeating α-(1→2)-L-rhamnose(Rha)-α-(1→4)-D-GalA disaccharide units. Also in the RG I backbone the GalA residues may be *O*-acetylated (Carpita and Gibeaut, 1993; Schols *et al.*, 1990).

Side-chains, mainly consisting of arabinan and/or galactan, may be attached to the RG I backbone at the *C*-4 position of the Rha residues (Carpita and Gibeaut, 1993; O'Neill *et al*., 1990; Schols and Voragen, 1994). To date, there is little evidence that arabinan and galactan occur as independent polysaccharides in the cell wall. The macromolecular structure composed of RG I, galactan and arabinan is often referred to as hairy regions (de Vries *et al*., 1981), with arabinan and galactan comprising the hairs. Of all potato cell wall components, pectin has most potential to be used in food industrial applications. Currently, mostly apple pomace and citrus peels, two by-products of food industrial processes, are used for pectin extraction because of their high degree of methylation and a low proportion of hairy regions (Voragen *et al*. 1995).

Even though the pectin and cellulose/xyloglucan networks seem to exist independently and fulfil different functions in the plant cell wall several studies have shown the compensatory ability of the pectin network upon cellulose depletion. Both tomato and tobacco suspension culture cells adapted to growth on cellulose synthase inhibitors showed increased levels of pectin, suggesting that a stronger Ca^{2+}-bridged network compensates for the missing load-bearing cellulose (Shedletzky *et al*., 1992; Wells *et al*., 1994). Additionally, the absence of cellulose in the wall resulted in secretion of xyloglucan molecules in the culture medium. A down regulation of cellulose levels in potato tubers is expected to result in increased pectin levels, consequently increasing the cell wall fraction most interesting for industrial applications.

The structure of the individual cell wall polysaccharides and affinity of these for each other are thus well described. Nevertheless, much less information is available on the assembly of these polysaccharides into higher order structures and on their specific functions in the cell wall. Before it is possible to bio-engineer viable plants with altered cell wall polysaccharide structures, which are improved for particular applications, it is necessary to understand the biosynthetic pathways for building and modifying them, in addition to their biological significance. In the following paragraphs several developmental studies, showing the naturally occurring variation in pectin structure which helped to clarify the biological significance of the different hairy region structures for cell wall architecture/properties and developmental processes are described. Additionally, also studies on mutagenized and genetically modified plants which showed the effect of alterations in the level or structure of cell wall polysaccharides (both pectin and cellulose) on plant development and cell wall structure as a whole are discussed.

THE SPATIAL DISTRIBUTION AND DEVELOPMENTAL REGULATION OF THE DIFFERENT CELL WALL POLYSACCHARIDES INDICATES THEIR SPECIFIC BIOLOGICAL SIGNIFICANCE

Plants exhibit species-specific differences in their cell wall structure, together with compositional variations in the pectic polysaccharides, and their relative abundance. Determination of the monosaccharide compositions of different plant polymers provides useful structural information about pectic polysaccharides, but this approach fails to consider the importance of tissue- and cell-specific localisations of particular pectins. The production of a series of antibodies recognizing different epitopes, each representing a part or particular structure of pectin, has clearly facilitated studies of developmental, tissue, and cell specific localization of the corresponding pectic structures (Knox, 1997; Willats et al., 2000; Willats et al., 2001a). The monoclonal antibodies LM5 (recognizing (1→4)-β-D-galactan; Jones et al., 1997), and LM6 (recognizing (1→5)-α-L-arabinan; Willats et al., 1998), have been used extensively to study the presence and location of their respective epitopes in different plants and tissues at different developmental stages. These experiments have clearly indicated that hairy regions appear in a tightly regulated fashion with respect to cell wall localization, and also in relation to cell differentiation and cell proliferation (Bush et al., 2001; Bush and McCann, 1999; Jones et al., 1997; McCartney et al., 2000; Orfila and Knox, 2000; Willats et al., 1998; Willats et al., 1999; Willats et al., 2000; Willats et al., 2001b).

In potato tubers, pectic epitopes are developmentally regulated during tuberisation and show also tissue-specific localisations in the mature tuber (Bush and McCann, 1999; Bush et al., 2001). An example of this are the labelling patterns of the LM5 and 2F4 (recognizing a calcium induced conformation of HGA; Liners et al., 1994) antibodies as shown in Figure 3. The distributions of epitopes of galactan and HGA are complementary. The LM5 and LM6 antibodies which both recognize RG I side-chains nevertheless show differences in labelling patterns. In stolons, the arabinan is enriched in the younger cells at the stolon tip (distal to the hook), while the galactan becomes more abundant in walls of older, elongated cells proximal to the stolon hook. In mature tubers, the arabinan epitopes are located throughout primary walls and middle lamellae of both cortical and perimedullary cell walls except at the expanded middle lamella at cell corners (Bush et al., 2001). Galactan is more abundantly localized in the primary walls of cortical and perimedullary tissues and in the primary cell wall it is mostly localized to the region close to the plasma membrane. In vascular walls and at pit fields of parenchymal walls, the LM5 epitope is greatly reduced in abundance, whilst the abundance of the LM6 arabinan epitope is reduced to a lesser extent in vascular walls and is not altered at pit fields (Bush and McCann, 1999). These labelling studies clearly show that in potato some co-localisation of the galactan and arabinan epitopes occurs. Nevertheless, the distinct distribution of these epitopes indicates the existence of different populations of RG I bearing different hairs with the implication that each type may influence the developmental characteristics of the wall.
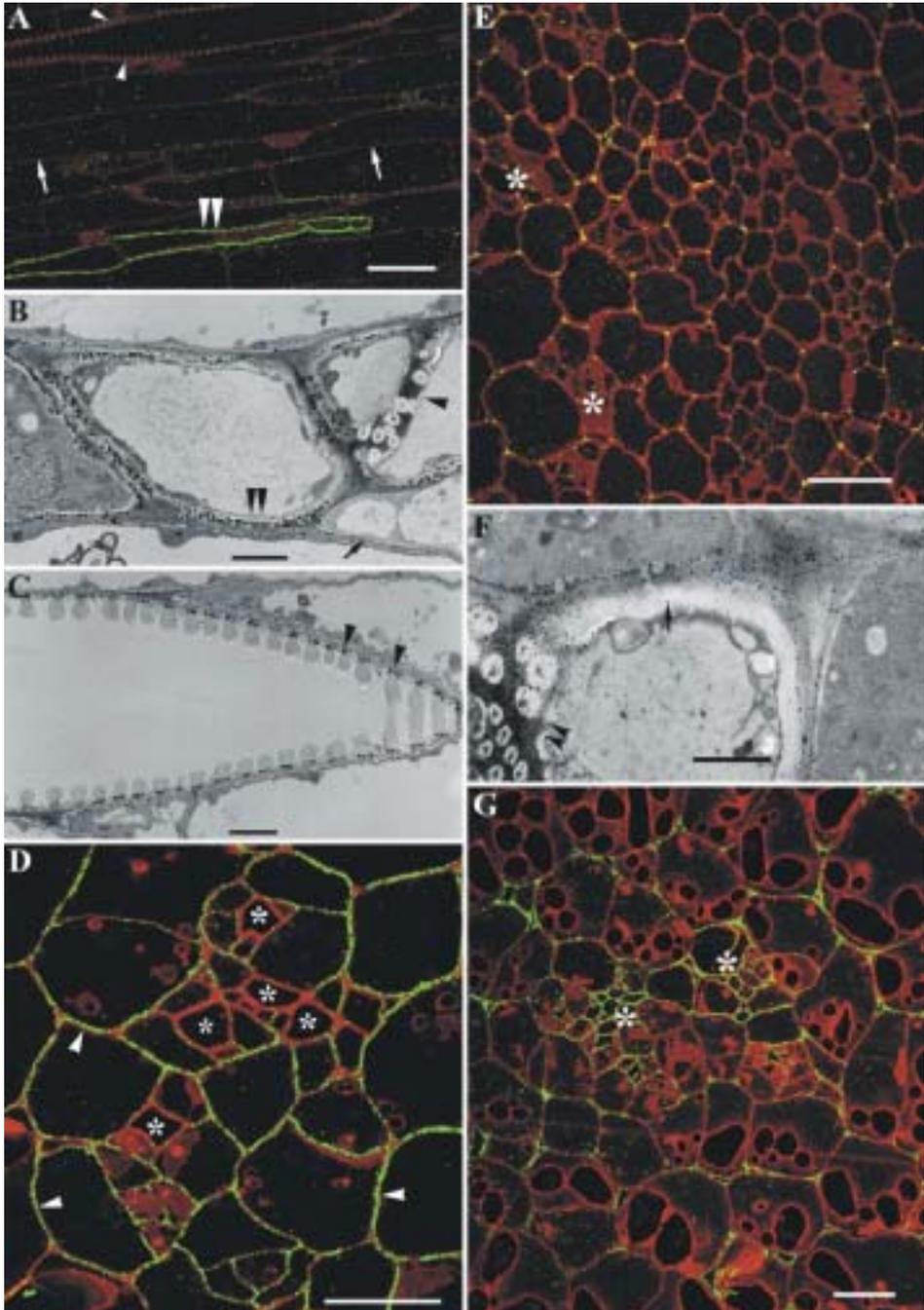
FIGURE 3.

Sections of vascular tissue from elongating stolons (A-C, E-F) and 32mm mature tubers (D, G) immunogold labelled and silver-enhanced with monoclonal antibodies LM5 (A-D) and 2F4 (E-G) and then imaged by reflection confocal laser scanning microscopy (A, D-E, G) and transmission electron microscopy (B-C, F). Monoclonal antibody LM5 recognises β-(1,4)-galactan in RG I hairs, whilst the 2F4 epitope is a calcium-induced conformation of HGA. The walls of phloem sieve tubes in stolons label strongly with LM5 (A-B, double arrowheads), whereas parenchymal cell walls label weakly (A, B, arrows). The LM5 epitope is present at the base of xylem secondary thickenings (A, C, single arrowheads), but not in phloem sieve plates (B, single arrowhead). In mature tubers (D), the LM5 epitope is absent in vascular tissue (D, *), but more abundant in parenchymal walls (D, arrowheads); this contrasts to the situation in stolons (A). The 2F4 epitope is more abundant in vascular tissue of mature tubers (G, *) compared to stolons (E, *). The phloem sieve plate is labelled by 2F4 (F, double arrowheads), whilst the 2F4 epitope is absent from the electron-lucent inner regions of the sieve tube walls (F, arrows). There is therefore a complementary developmental pattern of galactan and HGA epitope expression. Scale bars represent 20μm in A, D, G, 40μm in E and 2μm in B-C, F.

Galactans and arabinans of other plants also show differences in developmental and spatial localisation. McCartney and co-workers (2000) showed that, in pea cotyledon cell walls, both HGA and (1→5)-α-L-arabinan are continuously present, in contrast to a late developmental appearance of (1→4)-β-D-galactan. Further, the galactan was localised in the region of the cell wall close to the plasma membrane. This localisation of galactan is also found in other species (Ermel *et al.*, 2000; Freshour *et al.*, 1996; Jones *et al.*, 1997; Orfila *et al.*, 2001; Vicré *et al.*, 1998), and in most cases might be explained by deposition at a later stage of development (Bush and McCann, 1999; Vicré *et al.*, 1998). The developmental deposition of galactan in pea correlated with an increased firmness of the cotyledons (McCartney *et al.*, 2000). In tomato and kiwi, the loss of galactan is correlated with fruit softening and is expected to be an important trigger of additional cell wall changes associated with fruit ripening (Jones *et al.*, 1997; Redgwell *et al.*, 1997). The fact that these correlations were only found for the galactan and not for arabinan suggests that the different types of hairy regions have different functions in the cell wall, but it remains difficult to assign a specific characteristic of the wall or tissue to a particular pectic structure. The major reason for this is that during tissue development, other cell wall polymers will also be modified. The ability to produce plants with a specific, tailor-made, cell wall composition will be extremely useful to analyze the functions of individual cell wall polysaccharides further.

The generation, isolation and characterisation of cell wall mutants

More and more cell wall mutants are identified showing various alterations of the cell wall composition as a result from mutations in a whole range of genes. Since multiple steps and processes are involved in the development and maintenance of plant cell walls many different mutations can result in a modified cell wall composition. These processes for instance include the generation of substrate sugars and synthesis of the actual polysaccharides, but also the role of the secretory pathway for their final deposition in the extracellular space and for instance the *in muro* modifications and re-arrangements of the polysaccharides during growth and development of the cells. Most plants with a modified cell wall composition have been produced in *Arabidopsis thaliana* by a random mutagenesis approach, using chemicals, X-ray irradiation or T-DNA insertion (for an overview see Fagard *et al*., (2000a)). These mutants have been the starting point for the isolation and characterization of genes involved in the biosynthesis of cell wall polysaccharides. Many cell wall mutants described so far have been isolated based on altered growth (His *et al*., 2001). Identification of cell wall mutants is also possible by analysing the monosaccharide composition of isolated cell wall material, but this is a very laborious way to screen a large mutagenised population (Reiter *et al*., 1997). New methods, with minimal sample preparation, have been developed to screen directly for modified cell wall polysaccharides. Such methods will facilitate the identification of new mutants. Fourier transform infrared (FTIR) microspectroscopy has proven to be a powerful tool for the screening of cell wall mutants (Chen *et al*., 1998). Further, the various anti-pectin antibodies (Willats *et al*., 2000) can be used for the identification of mutants with a modified cell wall composition (Willats *et al*., 2001b).

A whole range of mutants has been described in which an altered pectin composition was identified as an effect of disturbed synthesis, modification or deposition. In particular cases the pectin modification was identified as a secondary effect of alterations in, for instance, the cellulose composition. A number of plants showing an altered cell wall composition as a result of mutations interfering at various stages of cell wall development and maintenance are shown in Table 1. The *Arabidopsis mur* mutants, as described by Reiter and co-workers (1997), were isolated by a screening based on monosaccharide composition analysis. Among these, *mur4, 5, 6* and *7* show a reduction of arabinose, which is due to a decrease in arabinan in either hairy regions or arabinogalactan-proteins (AGPs). The 50% reduction of arabinose in *mur*4 is too high to be accounted for solely by a reduction in AGP-arabinose. Further analysis of this mutant suggested a mutation in a membrane-bound UDP-D-xylose 4-epimerase, that blocks the conversion of UDP-D-xylose to UDP-L-arabinose (Burget and Reiter, 1999). The *mur8* mutant is reduced in rhamnose and the *mur10* has a modification in fucose, xylose and arabinose. The *mur11* mutants show modifications in rhamnose, fucose, xylose and mannose. Most of the *mur* mutants showed no visible phenotype; of

the *mur* mutants listed in Table 1 only *mur10* showed a reduction in growth rate and vigour.

TABLE 1. Mutagenized plants with a modified cell wall composition

Mutant	Pectin modification	Phenotype	Mutated gene	Reference
mur5-6-7	↓ Ara	not observed	?	Reiter *et al.* (1997)
mur4	Ara ↓ 50%	not observed	UDP-D-Xyl-4-epimerase	Burget and Reiter (1999)
mur8	↓ Rha	not observed	?	Reiter *et al.* (1997)
mur10	↓ Fuc and Xyl, ↑ Ara	slow growth, dark-green leaves, low seed-set	?	Reiter *et al.* (1997)
mur11	↓ Rha, Fuc and Xyl, ↑ Man	not observed	?	Reiter *et al.* (1997)
nolac-H14	abnormal localization of the pectin	has non-organogenic callus with loosely attached cells	?	Iwai *et al.* (2001)
Cnr	↓ de-esterified HGA, disrupted Ara deposition	disturbed fruit ripening, reduced cell-to-cell adhesion and non-swollen cell walls in the pericarp	?	Orfila *et al.* (2001)
emb30	abnormal localisation of the pectin	seeds unable to pass through normal embryogenesis, abnormal plants	similar to Sec7p, which functions in the secretory pathway	Shevell *et al.* (2000)
procuste1	↓ cellulose ↓ galactan	decreased cell elongation in roots & dark-grown hypocotyls	CesA6	Fagard *et al.* (2000b)
rsw1	↓ cellulose, ↑ pectin	shows a radial swelling upon growth at restr. temp of 31°C	CesA1	Peng *et al.* (2000)
korrigan	↓ cellulose, ↑ HGA, ↓ RG I with galactan hairs	Dwarfed growth	membrane-bound endo-1,4-β-glucanase	His *et al.* (2001)
acw1	↓ cellulose, ↑ pectin	Impaired cell elongation upon growth at restr. temp of 31°C	membrane-bound endo-1,4-β-glucanase	Sato *et al.* (2001)

The *emb30* (Shevell *et al*., 2000), *nolac-H14* (Iwai *et al*., 2001) and *cnr* (Orfila *et al*., 2001) mutants are some examples of plants in which the cell wall composition is altered by disturbed pectin deposition. The *Arabidopsis emb30* mutant (Shevell *et al*., 2000) has a mutation in a gene that is likely to be part of the secretory pathway. In these mutants the pectin shows an abnormal localisation, and the seeds are unable to develop into a normal plant which is most likely due to a lack of control of the polarity of cell divisions and expansions during embryogenesis. Another example is the *Nicotiana plumbaginifolia nolac-H14* mutant (Iwai *et al*., 2001) which is characterized by having a <u>n</u>on-<u>o</u>rganogenic callus with <u>l</u>oosely <u>a</u>ttached <u>c</u>ells. In contrast to wild-type callus, the mutant, which can only be maintained as a callus line, does not stain with ruthenium red (recognizing pectin) in the middle lamella and cell walls, and the pectin seems to be relocated to the surface of the callus and the growth medium. Sugar composition analysis, comparing the *nolac-H14* with wild-type callus, shows relative decreases in arabinose, xylose, galactose, glucose and mannose in whole cell extracts and relative increases of these sugars and galactose in the culture medium. These changes are likely to be linked to an aberrant deposition of pectin, although a modification in arabino-galactan proteins (AGPs) can not be excluded.

The *Cnr* ripening mutant of tomato also shows a disrupted deposition of several pectic polysaccharides (Orfila *et al.*, 2001). The mutant, for which the genetic basis has not yet been identified, has a pericarp with altered physical properties, including non-swollen cell walls and reduced intercellular adhesion (Thompson *et al.*, 1999). Even though no major differences were found by comparing the monosaccharide composition of the *Cnr* mutant and the wild type, antibody labelling clearly identified modifications in HGA and arabinan localization. A reduction of long de-esterified stretches of HGA possibly explains the reduced calcium-binding of the *Cnr* middle lamella, resulting in a reduced cell-to-cell adhesion. The additional disrupted deposition of $(1{\rightarrow}5)$-α-arabinan did not correlate to the altered cell wall properties (Orfila *et al,*. 2001).

The fact that alterations in the cellulose composition may also influence pectin structure was already indicated in experiments studying *in vitro* cultures of cells adapted to growth on cellulose synthase inhibitors. Tomato and tobacco cell lines adapted to growth on 2,6-dichlorobenzonitrile showed a 95% reduction in cellulose which was accompanied by increased levels in pectin which were suggested to compensate for the missing load-bearing cellulose by forming a Ca^{2+}-bridged network (Shedletzky *et al*., 1992). This effect was also found for several *Arabidopsis* lines in which a mutation in the gene encoding the catalytic subunit of the cellulose synthase complex (*CesA*) resulted in depleted cellulose deposition in primary walls. In the *procuste1* (*prc1*) mutant, harbouring a mutation in the *CesA6* gene, cellulose deficiency results in decreased cell elongation in roots and dark-grown hypocotyls of the plants (Fagard *et al*., 2000b). Apart from the decreased cellulose level, the pectic fraction of cell wall isolates showed reduced levels of galactose indicating a decrease in the number or length of the galactan side chains. Monosaccharide composition analysis of

the radial swelling mutant *rsw1* indicated slight increases in pectic components upon a larger decrease in cellulose as an effect of a mutated *CesA1* (Peng *et al.*, 2000). In the *korrigan* mutant (His *et al.*, 2001), a mutation in a membrane-bound endo-1,4-β-glucanase resulted in a reduction of the cellulose content. This reduction in cellulose was accompanied with an increase in HGA and a decrease in RG I with galactan hairs. Growth of mutants harbouring temperature-sensitive alleles of *KORRIGAN* at the restrictive temperature also showed cellulose reductions to 40% accompanied with increased pectin content to 162% (Sato *et al.*, 2001). These modifications in pectin structure resulting from depleted cellulose levels show the plasticity of the cell wall and suggest the existence of feedback mechanisms to maintain the important properties of the wall.

GENETIC MODIFICATION TO GENERATE PLANTS WITH AN ALTERED CELL WALL COMPOSITION

Even though the generation of cell wall mutants has proven a very useful approach to clarify the varying factors participating in the final cell wall composition, the generation of novel mutants remains a random process. In crop plants, the generation of mutants can be extremely difficult due to their polyploidy. An alternative here is genetic modification, which is possible in a large number of crop plants. Further, the down or up-regulation of an endogenous gene or the introduction of a heterologous gene provides a direct approach to specifically modify a known cell wall polysaccharide structure. Ideally, one would like to manipulate the biosynthetic machinery of the individual polysaccharides, but unfortunately at the start of the work described in this thesis only few genes involved in wall polysaccharide biosynthesis had been cloned (Mohnen, 1999). A number of plants in which altered cell wall structures were obtained by genetic modification are listed in Table 2.

In relation to altering pectin structure most studies have made use of pectin modifying or degrading enzymes. Some examples are found in studies aimed to manipulate the processes causing fruit ripening and softening (reviewed by Brummell and Harpster, 2001). In these experiments, the expression of several genes (e.g. pectin methyl esterases and *endo*-polygalacturonases) responsible for modification of the pectin structure were down-regulated. An important modification of the hairy regions during fruit ripening is the decline in pectic galactan early in the ripening process (Brummell and Harpster, 2001). Suppression of the TBG4 gene, encoding a β-galactosidase activity (Smith and Gross, 2000) in tomato, showed a reduced fruit softening (Brummell and Harpster, 2001). This suggested that RG I galactan chains contribute to maintaining the firmness of the fruit, and that their degradation by endogenous enzymes is one of the changes leading to fruit softening during ripening.

Table 2. Genetically modified plants with an altered cell wall composition

Gene	Plant	Modification	Phenotype	Reference
* *TBG4*	tomato	no decrease in galactan	delayed and reduced fruit softening	Brummell and Harpster (2001)
** e*GAL*	potato	Gal ↓	not observed	Sørensen *et al.* (2000)
** eG*ARA*	potato	Ara ↓	not observed	Skjøt *et al.* (2002)
* *UDP-Glc-4-epimerase*	*Arabidopsis*	no galactan ↑ during growth on galactose medium	not observed	Dörmann and Benning (1998)
* *CesA*	tobacco	↓ cellulose ↑ HGA	Dwarfed, swelling of leaf cells in the epidermis	Burton *et al.* (2000)
* *CesA1*	*Arabidopsis*	cellulose ↓	shorter stems, and smaller leaves and floral organs	Burn *et al.* (2002)
*		= anti-sense expression of an endogenous gene		
**		= sense expression of a heterologous gene		

Sørensen *et al*. (2000) and Skjøt *et al*. (2002) showed the possibility to generate potato plants with altered cell wall polysaccharide structures by the introduction of a heterologous gene encoding a pectin degrading enzyme. Potato plants expressing the *endo*-galactanase (*e*GAL) from *Aspergillus*, able to degrade (unsubstituted) 1→4-β-D-galactan to galactose and galactobiose (Christgau *et al*., 1995), showed a specific removal of part of the galactan sidechains attached to the RG I (Sørensen *et al*., 2000). The Golgi-targeted expression of an *endo*-arabinanase (*e*GARA) from *Aspergillus*, which hydrolyzes α-1→5-L-arabinan (Beldman *et al*., 1993; Skjøt *et al*., 2001), resulted in potato tubers with RG I with a low level of arabinosylation (Skjøt *et al*., 2002). These experiments showed that depletion of neither galactan nor arabinan side chains of the RG I had an effect on the composition of other cell wall polysaccharide structures or plant developmental characteristics. This suggests that individual removal or absence of the two types of RG I hairs alone will not result in direct modifications of other structures or biological events.

An alternative direct approach to obtain a modified cell wall composition in plants is by manipulation of the biosynthetic machinery. One example of such a biosynthetic gene is the UDP-Glc-4-epimerase, which was used for sense and antisense expression in *Arabidopsis* (Dörmann and Benning, 1998). The enzyme, which catalyzes the reversible epimerization of UDP-galactose to UDP-glucose, is important in maintaining the pool of UDP-galactose which is the building block for the galactan hairs. The sense and antisense expression resulted in 3-fold increases and 90% reduction of the corresponding enzyme activity, respectively. Nevertheless, this did not

result in a modified composition of the pectin during normal growth of the plants. Growth of the *Arabidopsis* plantlets on agar containing galactose, increased the amount of cell wall bound galactose in both wild type as well as in antisense plants with a decreased UDP-Glc-4-epimerase activity. In plants with an increased epimerase activity this effect was not found. This suggests that the UDP-Glc-4-epimerase is indeed important for maintaining the UDP-galactose pool and indirectly influences the amount of cell wall bound galactose. Nevertheless, in these transformants, enzyme levels as low as 10% of wild type are still sufficient to maintain normal UDP-galactose levels. Another example of direct interference with cell wall biosynthesis concerns the down regulation of cellulose synthase (*CesA*) genes. Currently this is the only wall polysaccharide synthase for which plants with decreased expression levels have been generated. Burton and co-workers (2000) showed the efficient use of the virus-induced gene silencing (VIGS) system to down regulate *CesA* expression levels in *Nicotiana benthamiana*. These plants showed a "dwarfed" phenotype (shorter internode lengths and smaller leaves) and in the epidermis of infected leaves cells were swollen, probably as an effect of the reduced cellulose content. In addition to the 25% reduction of cellulose in these plants, a 45% increase of homogalacturonan was observed in addition to a decrease in esterification of the pectic polysaccharides. Also in *Arabidopsis* cellulose synthesis was down regulated by antisense expression of three *CesA* (1, 2 and 3) genes (Burn *et al.*, 2002). Antisense expression of the *CesA1* (mutated in the *rsw1* mutant; Arioli *et al.*, 1998) and *CesA3* generated a similar but milder and not completely identical phenotype in comparison with the *rsw1* mutant, showing e.g. shorter stems and smaller leaves and floral organs compared to WT plants. *CesA2* showed only a mild phenotype of reduced elongation rate when grown at 31°C. In the respective transformants only very small decreases in cellulose could be identified. As to be expected from the large gene family of *CesA* genes these experiments indicate that multiple cellulose synthases are involved in cellulose biosynthesis.

OBJECTIVES AND OUTLINE OF THIS THESIS

In this thesis several approaches to obtain an altered cell wall composition in potato by genetic modification have been studied. The alterations were specifically aimed to generate potato plants having tubers with a modified pectin composition. Many questions concerning the biological significance of individual cell wall polysaccharides (pectin in particular) and their interaction have not been answered. The generation of genetically modified plants with specific modifications in these structures will be a useful tool in answering some of these questions. In addition, some *in planta* modifications of potato pectin structure may improve pectin characteristics resulting in enhanced suitability for food and non-food industrial applications. This will enhance the economical value of the potato fibre, which is a voluminous by-product of the starch industry.

Three different approaches to generate transformants with a modified cell wall composition have been examined. (i) The specific degradation of whole or parts of cell wall polysaccharides which are already deposited in the cell wall. This can be achieved by the heterologous expression of wall polysaccharide degrading enzymes from for instance fungi. (ii) The modulation of expression for enzymes which play an important role in maintaining the pools of nucleotide sugars. It is expected that alterations in the nucleotide sugar levels, of those which are incorporated in cell wall polysaccharides, will reflect on the final cell wall composition. (iii) Decreasing or increasing the expression levels of synthases responsible for the polymerisation of the varying backbone structures. Even though the latter two approaches seem more efficient, their applicability is limited due to the low availability of isolated genes involved in biosynthesis of cell wall polysaccharides (especially at the start of this thesis work). Therefore an RNA fingerprinting experiment was started to study the generation of cell walls by potato leaf protoplasts and the possibility to identify new genes involved in this very complex process.

Chapter 2 describes the introduction of a RG I lyase (*e*RGL) from *Aspergillus aculeatus* (Kofod *et al.*, 1994; Mutter *et al.*, 1996; Mutter *et al.*, 1998) in potato. This enzyme is able to degrade the RG I backbone resulting in an *in muro* fragmentation of the hairy region of the pectin. The effect of this *e*RGL expression was studied by microscopic analysis using pectin specific antibodies and on the level of its monosaccharide composition.

One of few isolated genes involved in cell wall biosynthesis are the cellulose synthase (*CesA*) genes. Apart from the expected effects on cellulose levels upon modulating *CesA* expression levels, this most likely also affects pectin structures as shown in several suspension cultures, mutants and transgenic plants with depleted cellulose. Chapter 3 describes the isolation of 4 *CesA* cDNA clones from a potato tuber cDNA library. One full length clone was used in sense and antisense constructs to study the possibility, and effects, of increasing and decreasing cellulose levels on potato plants and the overall cell wall composition. Additionally, transgenic plants were generated in which the individual potato *CesA* clones were specifically down regulated (Chapter 4). Therefore, the Class Specific Region (CSR), a specific region of the gene important in revealing sub-classes of the *CesA* family members, was used for antisense expression. All so-called cellulose synthase transformants were initially analysed by Fourier Transform Infra-Red (FTIR) microspectroscopy to identify those transformants with an altered cell wall composition. Subsequently the cellulose content and monosaccharide composition of the cell wall polysaccharides was studied.

Another point of interference in cell wall synthesis that was studied concerned the generation and/or maintenance of the nucleotide sugar precursors for cell wall polysaccharides. Chapter 5 describes the isolation of two UDP-Glc 4-epimerases (UGE) from a potato tuber cDNA-library. The effect of increasing the expression of this enzyme, important for maintaining the UDP-galactose pool size, on the final

amount of cell wall bound galactan was studied by individually overexpressing the two *UGEs*.

Future possibilities to generate transgenic plants harbouring specific cell wall structures will obviously benefit from the isolation of new genes involved in cell wall polysaccharide synthesis. In chapter 6 we describe the investigation of using an RNA fingerprinting study on potato leaf protoplasts to identify candidate genes important for these processes. At different time-points during the process of cell wall biosynthesis RNA expression was visualised using cDNA-AFLP after which several transcripts were isolated and sequenced to verify their possible function during wall development.

In chapter 7, the outcome of the different approaches to obtain modified cell wall composition, the limits of these modifications, and the applicability of these plants for industrial purposes and answering biological questions is discussed.

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2

In muro fragmentation of the rhamnogalacturonan I backbone in potato (*Solanum tuberosum* L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development

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SUMMARY

Rhamnogalacturonan (RG) I is a branched pectic polysaccharide in plant cell walls. Rhamnogalacturonan lyase (*e*RGL) from *Aspergillus aculeatus* is able to cleave the RG I backbone at specific sites. Transgenic potato (*Solanum tuberosum* L.) plants were made by the introduction of the gene encoding *e*RGL, under control of the granule-bound starch synthase promoter. The *e*RGL protein was successfully expressed and translated into an active form, demonstrated by *e*RGL activity in the tuber extracts. The transgenic plants produced tubers with clear morphological alterations, including radial swelling of the periderm cells and development of intercellular spaces in the cortex. Sugar compositional analysis of the isolated cell walls showed a large reduction in galactosyl and arabinosyl residues in transgenic tubers. Immunocytochemical studies using the LM5 (galactan) and LM6 (arabinan) antibodies also showed a large reduction in galactan and arabinan side-chains of RG I. Most of the remaining LM5 epitopes were located in the expanded middle lamella at cell corners of *e*RGL tubers, which is in contrast to their normal location in the primary wall of wild type tubers. These data suggest that RG I has an important role in anchoring galactans and arabinans at particular regions in the wall and in normal development of the periderm.

INTRODUCTION

The primary cell wall is composed of several polysaccharides (90%) and some structural proteins (10%) (McNeil *et al*., 1984). Of these polysaccharides, cellulose and cross-linking glycans form a load-bearing network that is embedded in a matrix of pectic polysaccharides (Carpita and Gibeaut, 1993). The pectic molecules comprise mainly homogalacturonan (HGA) and rhamnogalacturonan I (RG I). HGA is composed of unbranched α-1,4-linked galacturonic acid (GalA) residues. RG I has a backbone composed of repeating α-(1,2)-L-rhamnose(Rha)-α-(1,4)-D-GalA disaccharide units. Side-chains, mainly consisting of arabinan and/or galactan, may be attached to the RG I backbone at the C-4 position of the Rha residues (Carpita and Gibeaut, 1993; O'Neill *et al*., 1990; Schols and Voragen, 1994). The GalA residues in the HGA and RG I backbone may be methyl-esterified and/or O-acetylated (Carpita and Gibeaut, 1993). The functions of the individual pectic polysaccharides are still unclear. They have been implicated in regulating wall porosity, cell separation, cell expansion, organogenesis, textural changes during fruit ripening and as a source of oligosaccharins which can act as signalling molecules (Aldington and Fry, 1993; Darvill *et al*., 1992; McCann and Roberts, 1994). The presence and composition of the pectic polysaccharides has been shown to depend on the plant tissue, and the position in the cell wall, but also on the developmental stage of the plant (tissue) (reviewed by Willats *et al*., 2001). These observations support the idea of specific functions for the different pectic polysaccharides in plant cell walls.

Pectin can be modified *in planta* by mutagenesis (Reiter *et al*., 1997), but this is difficult to achieve in potato because it is a tetraploid crop. A more direct approach is to modify pectin *in planta* by the apoplastic expression of genes encoding glycanase activities (Vincken *et al*. 1999). Whilst many potential pectin-modifying enzymes are annotated in the *Arabidopsis* database, very few of these have been authenticated by demonstration of a corresponding biochemical activity. However, recombinant glycanases from fungi have well-characterised activities on cell-wall substrates. The introduction of an endo-galactanase from *Aspergillus aculeatus* in potato has been shown to result in the specific removal of part of the galactan sidechains attached to the RG I (Sørensen *et al*., 2000). Tubers expressing the endo-galactanase developed normally. In this paper, we report the consequences of fragmenting the RG I backbone rather than the attached side-chains.

We have generated potato plants over-expressing *Aspergillus* RG I lyase (*e*RGL: Kofod *et al*. (1994); Mutter *et al*. (1996); Mutter *et al*. (1998)), driving expression with the granule bound starch synthase promoter, which is highly active in potato tubers (Visser *et al*., 1991). All transformants were analyzed for RNA expression and the presence and activity of the introduced enzyme in tuber extracts. Furthermore, the effect on cell wall composition was analyzed by determining the monosaccharide composition, immunogold labelling and with Fourier Transform Infrared (FTIR) Microspectroscopy. Our results provide evidence that the side-chains of RG I become reduced in abundance and abnormally localised to the middle lamella at cell corners of transgenic tubers. Further, a phenotype of abnormal periderm development is observed, perhaps a consequence of abnormal cell division and cell swelling. This approach provides useful tools with which to evaluate the functions of pectin *in planta*.

RESULTS

*e*RGL transgenic plants make tubers with altered morphology

A construct was made to introduce the *e*RGL from *A. aculeatus* into potato tubers via *Agrobacterium tumefaciens*-mediated plant transformation. The granule bound starch synthase promoter was used to obtain a high expression in potato tubers. Twenty-one individual transformants were obtained and transferred to the greenhouse for tuber production. Compared to wild type (WT), the transformants showed characteristic differences. The transformants grew slower and their tubers were slower to produce a new plant after planting in soil. Crossing experiments demonstrated that the *e*RGL transgenic plants, in contrast to WT plants, produce only a limited number of flowers and have a low male fertility.

The *e*RGL plants produced tubers with a clearly altered morphology (Figure 1). These tubers were smaller compared to WT, had deeper 'eyes' and the tuber surface had a wrinkled appearance. This phenotype was observed in plants with a high (#9) *e*RGL expression, but also in plants with a (relatively) low (#18) expression of the gene.

Compared to the low *e*RGL expressers, the high expressers showed a more severe tuber phenotype. The average yield (grams of tuber material per plant) of the *e*RGL transformants was comparable to wild type due to a higher number of smaller tubers produced by each plant.
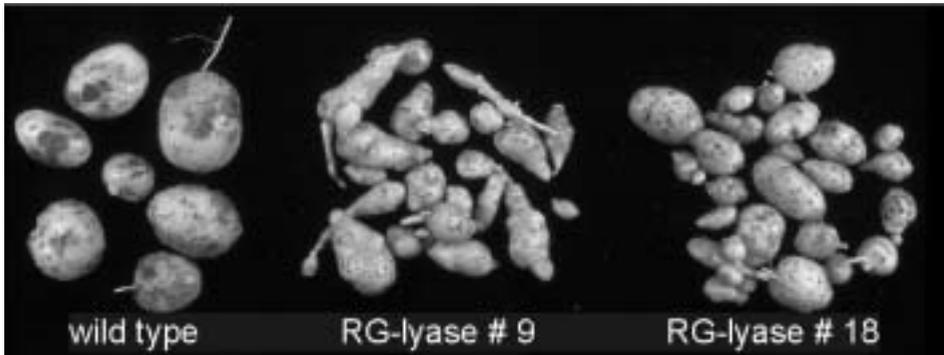
FIGURE 1.
A selection of tubers expressing the *e*RGL and a wild type control.
The two transformant clones, #9 and #18, show a high and low RNA expression of the *e*RGL respectively.

*e*RGL transformants express the transgenic RNA

DNA was isolated from the leaves of transformed plants and used for Southern analysis. In all plants, which were used for further analysis, the transgene could be detected. Furthermore, the number of T-DNA insertions was shown to vary from 1 to 4 (data not shown). Tubers were harvested and used for RNA isolation followed by Northern blot analysis (Figure 2a,b). A partial cDNA probe, corresponding to the introduced gene, was used with a 28S ribosomal RNA probe as a control (Figure 2b). In all transformants, RNA expression of the transgene could be detected in the potato tubers and a number of high and low expressers were identified (Figure 2a).

Transgenic enzyme activity: *e*RGL activity correlates to its level of RNA expression

Transgenic and wild type tuber extracts were made and subsequently measured for *e*RGL activity. Tuber extracts were added to saponified (saponification removes acetyl groups which inhibit the enzyme activity) RG from apple (MHR-S; Schols *et al.*, 1990b), and after 15 min and 1 hour incubations, degradation of the MHR-S was analyzed by high performance size exclusion chromatography (HPSEC). Extracts from WT tubers had no effect on the HPSEC elution pattern, but the *e*RGL transformants were able to degrade the polymeric population of apple RG-S into oligomers, as seen by a shift in the molecular weight distribution (Figure 2c). After 1-hour incubations, all extracts from the different *e*RGL transformants gave the same degradation pattern.

FIGURE 2.

Northern blot analysis and *e*RGL enzyme activity determination in WT and *e*RGL transformants.

a,b For all transformants total tuber RNA (40 µg per lane) was separated by gel electrophoresis, transferred onto a nylon membrane and probed using the *e*RGL (a) and ribosomal RNA (b) ^{32}P-labelled cDNAs. **c** HPSEC elution patterns of the degradation of a saponified apple RG preparation by different potato tuber extracts. *Blocked Grey line* is representative for incubations with WT extracts and inactivated *e*RGL extracts. *Black line e*RGL #9, 15 min incubation. *Circled Grey line e*RGL #9, 60 min incubation. *Blocked black line e*RGL #18, 15 min incubation. The peaks from retention time 32 min onwards result from salts and other impurities present in the crude protein extracts. **d** Based on the results as shown for *e*RGL transformants #9 and #18 in panel c, the relative enzyme activity of these and other transformants is expressed as +++ for the highest activity and – for no detectable activity.
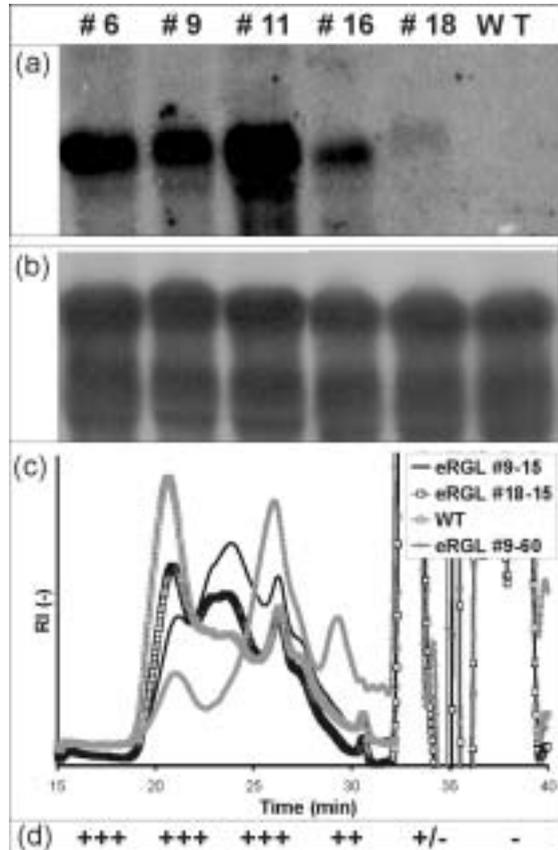
However, the 15 min incubations showed varying enzyme activity levels among the different transformants; the MHR-S populations were clearly further degraded upon treatment with the *e*RGL #9 extract than with the *e*RGL #18 extract. Furthermore, the level of *e*RGL enzyme activity for these transformants is clearly correlated to the RNA expression levels determined by Northern blot analysis (Figure 2a). Transformants #9 and #18 were subsequently marked as high and low expressers respectively (Figure 2d). The data above are in line with a rapid screening of WT and transgenic tuber cell walls by FTIR microspectroscopy (data not shown), which demonstrated that complex changes had occurred in the transgenic walls.

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Monosaccharide analysis shows gross changes in the composition of *e*RGL cell walls

Cell wall material (CWM) was isolated from WT and *e*RGL expressing tubers (transformant #9). Comparison of sugar composition from the *e*RGL transformants with WT showed some clear changes in the different cell wall components (Table 1). The *e*RGL #9 transformant has a markedly lower galactose (4.9 and 21.4 mol%, for *e*RGL #9 and WT plants respectively) and arabinose content (5.2 and 8.6 mol%, for *e*RGL #9 and WT plants respectively). These decreases in galactose and arabinose are in line with the decrease in rhamnose (0.9 and 1.4 mol%, for *e*RGL #9 and WT plants respectively), one of the backbone residues of RG I. Finally, this transformant shows an increase in the uronic acid content (37.0 and 22.6, for *e*RGL #9 and WT plants respectively). The sugar composition of *e*RGL #18 CWM showed similar, but less pronounced changes as that of *e*RGL #9 when compared to WT CWM (data not shown).

TABLE 1. Glycosyl residue composition (mol%) of the cell wall material isolated from tubers of wild type and rhamnogalacturonan lyase expressing potato plants. Values represent the average of a triplicate analysis ± the variance.

	WT	*e*RGL #9
Rha	1.4 ± 0.11	0.9 ± 0.25
Ara	8.6 ± 0.10	5.2 ± 0.11
Xyl	2.4 ± 0.13	4.4 ± 0.07
Man	1.8 ± 0.04	2.5 ± 0.09
Gal	21.4 ± 1.83	4.9 ± 0.05
Glc	41.8 ± 0.26	45.1 ± 0.22
uronic acid	22.6 ± 2.12	37.0 ± 0.28

WT and *e*RGL CWM have different extraction characteristics

The isolated CWM from WT and *e*RGL #9 was sequentially extracted with a series of solvents to determine: (i) if the various polymers from WT and *e*RGL #9 CWM have different extraction characteristics, and (ii) to obtain fractions that are enriched in RG I with side chains. Table 2 shows that the extraction efficiency was good because there were only small quantities of sugars left in the final residue apart from Glc which is celllulose. As expected, the chelator soluble solid (ChSS) and dilute alkali soluble solid (DASS) extracts contain mainly pectic polysaccharides, whereas the 1M alkali soluble solid (ASS) extract was rich in xyloglucan and some (arabino)galactan. Typically, the 6M ASS fractions are rich in pectic polysaccharides (besides xyloglucan). The pectin-containing fractions (ChSS, DASS, 6M ASS) of *e*RGL #9 tuber CWM are distinguished from those of WT tuber CWM by their much lower galactosyl and much higher uronosyl content. This is in accordance with the data in Table 1.

Figure 3a indicates the proportion of the various polysaccharides extracted from the total WT and *e*RGL #9 CWM. The most important conclusion from these data is that

the RG I-rich 6M ASS fraction from both WT and *e*RGL #9 potato tuber CWM (52 and 34%, respectively) forms an important part of the cell wall. The diagrams indicate that polymers are easier extracted from the transgenic CWM than from WT, because more material is recovered with the relatively mild extractants in the case of *e*RGL #9.

TABLE 2. Glycosyl residue composition (mol%) of various extracts from cell wall material isolated from tubers of wild type and rhamnogalacturonan lyase expressing potato plants. The values represent the average of a duplicate analysis. All extracts have been corrected for the presence of starch as detailed in the section experimental procedures.

	Rha	Ara	Xyl	Man	Gal	Glc	uronic acid
WT-ChSS	3.7	13.7	2.1	3.2	52	4.2	21.0
WT-DASS	2.9	11.6	2.9	10.2	43.5	5.8	18.9
WT-1M ASS	0	8.8	19.3	3.3	27.5	38.5	2.2
WT-6M ASS	4.6	16.8	4.1	2.6	50	8.2	13.8
WT-residue	0.5	1.8	1.8	1.4	1.5	84.7	8.4
*e*RGL #9-ChSS	1.8	5.7	1.8	2.2	9.8	3.6	75.2
*e*RGL #9-DASS	4.3	12.8	5.0	5.0	14.2	9.9	58.9
*e*RGL #9-1M ASS	0	12.4	23.7	5.7	6.7	47.4	3.6
*e*RGL #9-6M ASS	3.1	14.6	13	7.8	15.1	26	20.8
*e*RGL #9-residue	0	2.4	0.7	1.2	3.9	87.1	4.5

FIGURE 3.

a Pie charts showing the amount (% w/w) of polymers, sequentially extracted from cell wall material of wild type and transgenic potato tubers using different solvents. **b** HPAEC elution patterns of 6M ASS extracts from wild type and transgenic potato tuber cell wall material, which was treated with a fungal *e*RGL. Diagnostic oligosaccharides are indicated by n=1-4, and were identified by comparison of their retention times to those of a digest with known composition (Mutter *et al.*, 1998). The structure of the repeated oligomeric moiety is shown; Squares indicate Rha residues; circles, GalA residues; concentric circles, unsaturated Δ4,5-GalA residues; triangles, Gal residues. The control digests (6M ASS material incubated with inactivated enzyme) have a similar appearance as the lower line. For clarity, these have been omitted from the figure.
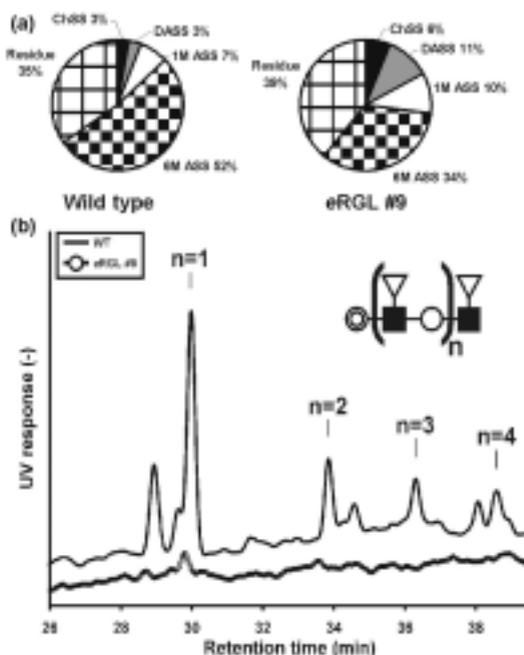
RG I is degraded in the transgenic *e*RGL #9 tubers

In order to investigate if the *e*RGL had fully degraded its substrate in the transgenic tuber walls, isolated CWM of WT and *e*RGL #9 tubers were treated *in vitro* with a fungal *e*RGL and the resulting digest analysed for diagnostic RG I oligomers by HPAEC. Release of oligosaccharides was not observed in this experiment, probably because the fungal *e*RGL could not access its substrate in the isolated CWMs. Therefore, the 6M ASS extracts, which are large fractions, rich in RG I, were treated with the fungal *e*RGL. Figure 3b clearly shows that the fungal *e*RGL releases the typical, galactosylated RG oligosaccharides only from the WT-6M ASS material (Mutter *et al.*, 1998). In contrast, only traces of oligosaccharides were released from the transgenic 6M ASS fraction, indicating that the *e*RGL substrate was depleted *in muro* from the transgenic tuber walls during development. Incubation of the extracts with inactivated fungal *e*RGL yielded a chromatogram comparable to that of *e*RGL #9. These results demonstrate that the majority of the RG I backbones were degraded in the transgenic potato tubers.

_e_RGL expressing tubers show a developmental phenotype

Comparison of toluidine-blue stained sections of WT and *e*RGL-expressing tubers, showed differences in the periderm and cortex (Figure 4). The normal appearance of the WT periderm is that of ordered layers of cells (generated by periclinal divisions) which become flattened and suberized as they mature (Figure 4a). The underlying cortex is arranged into roughly concentric rows of cells containing few starch granules and intercellular spaces (Figure 4a). In contrast, the periderm of the high *e*RGL-expressing tubers (#9) is disorganized (Figure 4b): there is a complete absence of ordered cell layers, due to the random division planes and expansion of the periderm cells. The periderm of the low expressing transformants (#18, data not shown) is more similar to WT, except that the cells in the peripheral layers expand isodiametrically. Compared to WT tubers, both high and low *e*RGL-expressers show marked increases in the number and size of cortical intercellular spaces (Figure 4b), but the perimedullary and vascular tissues exhibit a WT appearance.

FIGURE 4.

Micrographs of toluidine-blue-stained 0.5 µm-thick resin sections of wild type (a) and high *e*RGL (#9) expressing (b) tubers.

The wild type periderm (Pdm) is composed of regularly arranged flattened cells, but has poor sectioning qualities (asterisks indicate tears in the section); in contrast the transgenic periderm consists of randomly arranged swollen cells. Large intercellular spaces (arrowheads), which are absent in the wild type tuber, are a characteristic feature of the transgenic cortex (Ctx). Scale bar represents 0.5 mm; Pmed, perimedullary tissue.
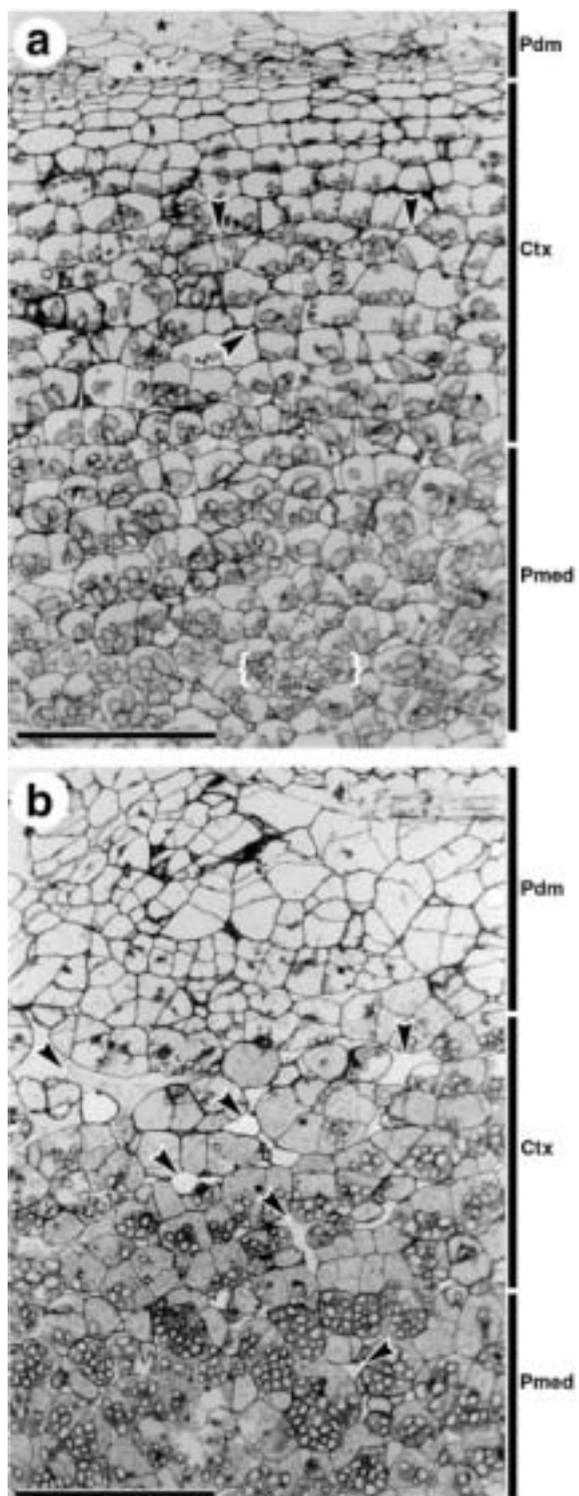
Galactan and arabinan epitopes are reduced in abundance and appear at altered locations in *e*RGL tuber walls

The mAb that recognizes Sycamore RG I, CCRC-M2 (Puhlmann *et al*., 1994), does not cross-react with potato (Bush and McCann, 1999). However the mAbs LM5 and LM6 recognize (1→4)-β-D-galactan and (1→5)-α-L-arabinan respectively, which are components of RG I side-chains and hence are indirect markers of the RG I backbone. We used reflectance confocal laser scanning microscopy (CLSM) and transmission electron microscopy to image immunogold-labelled sections of potato tuber from WT and *e*RGL transformants #9 and #18. The distribution of the LM5 and LM6 epitopes is well documented in WT tubers (Bush *et al*., 2001; Bush and McCann, 1999). Essentially, the WT periderm shows gradients of both epitopes (most abundant towards the cortex), the parenchymal cell walls throughout the tuber label strongly with both mAbs, whilst the vascular walls show relatively lower abundancies of both epitopes (Figure 5). The low and high expressing *e*RGL tubers gave similar labelling results to each other with both mAbs: the periderm walls labelled similarly to WT (compare Figure 5a, c with e, g, i and k). However, in low *e*RGL-expressers, galactan and arabinan epitopes were detected in the walls of the outer cortical cells, but deeper into the cortex, they were restricted to just a few cell corners (Figure 5e and g); in high *e*RGL-expressing tubers, they only occurred at a few cortical cell corners (Figure 5i and k). In the perimedullary tissue that comprises the bulk of the tuber, both epitopes were only detectable in the walls of the vascular tissue where the labelling intensities were unaffected and comparable to WT levels (compare Figure 5b, d with f, h, j and l).

In the electron microscope, it is clear that in WT tubers, the galactan epitope is restricted to the primary wall of parenchymal cells and absent from the middle lamella, whilst the arabinan epitope occurs throughout the walls, but both epitopes are absent from the expanded middle lamella at corners (Figure 6a and b).

In all *e*RGL transformants, both galactan and arabinan epitopes are greatly reduced throughout parenchymal walls, except in vascular cell walls (Figure 6c). The labelling of cell corners with mAb LM5 seen by CLSM is due to an altered location of the epitopes from their normal WT location in the primary wall to the expanded middle lamella or lining of intercellular spaces at cell corners (Figure 6d and e). The tuber cells showed characteristic features that were absent from WT cells: these were the increased abundance of biosynthetic organelles (Figure 6e) and the presence of a diffuse cytoplasmic label.

FIGURE 5.

Confocal scanning laser microscope images of wild type and *e*RGL expressing (#9 high expresser, #18 low expresser) tubers immunogold-labelled with LM5 (upper panel) and LM6 (lower panel) that recognize 1,4-galactan and 1,5-arabinan epitopes, respectively.

Images a, c, e, g, i and k show periderm (asterisk) and cortical (ctx) tissues, whilst b, d, f, h, j and l show vascular tissue (bracketed) from the perimedullary region of the tuber. Wild type cell walls (arrowheads) of starch parenchymal cells contain abundant galactan (a, b) and arabinan (c,
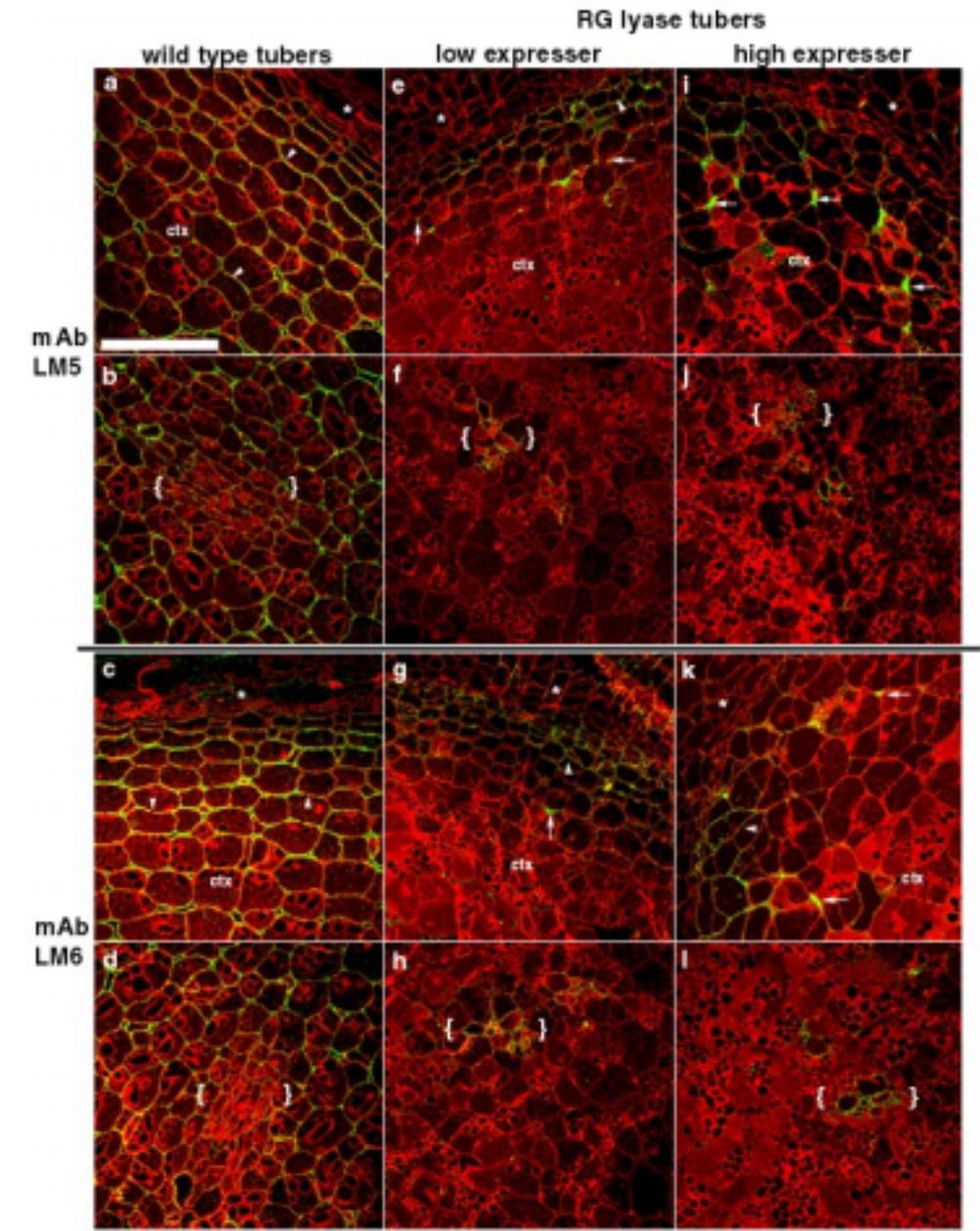
d) epitopes, the vascular walls are less enriched in these components (brackets in b and d). These epitopes are barely detectable in most starch parenchymal cell walls from the transgenic tubers (e-l), notable exceptions are some cells from the outer cortex (arrowhead in e, g and k) and the vascular walls (f, h, j and l), of which the latter ones show labelling intensities similar to the WT situation. LM5 and LM6 strongly label a few cell corners in the transgenic cortical tissues (arrows in e, g, i and k). Scale bar is 200 µm and applies to all images.
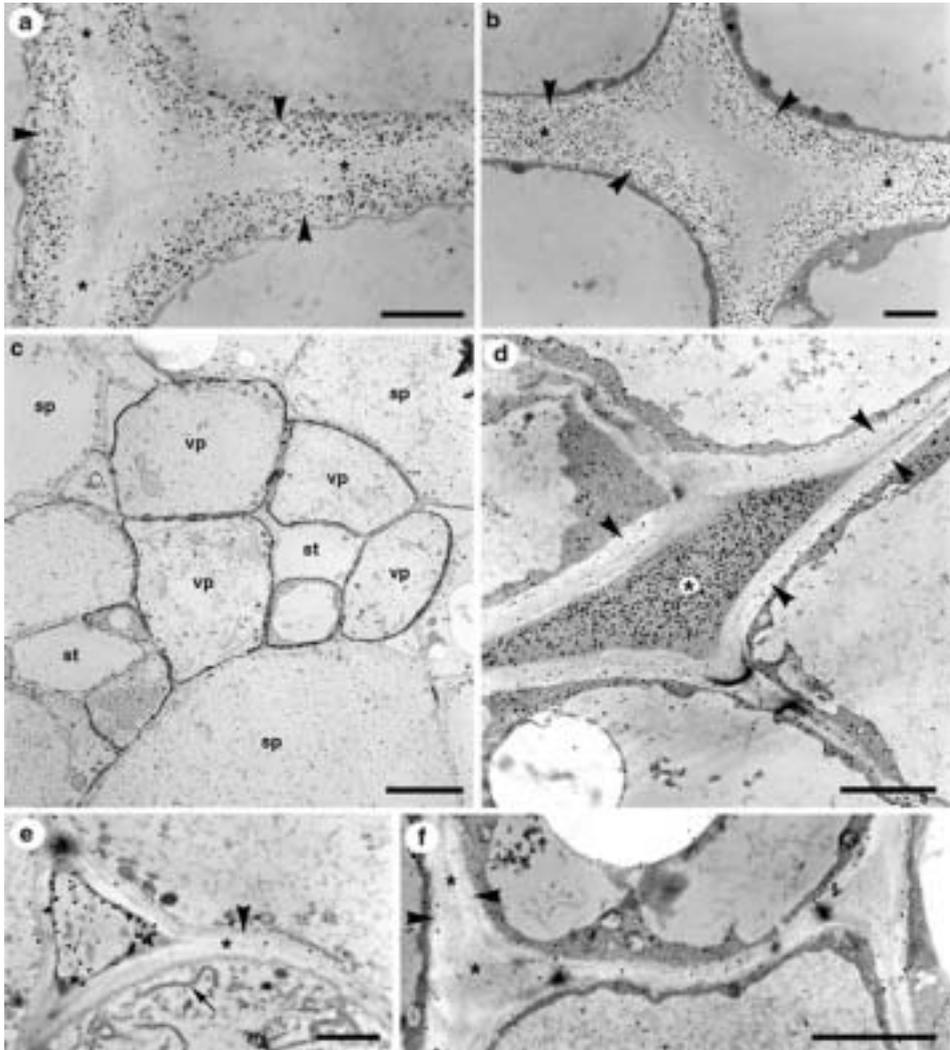
FIGURE 6.

Transmission electron micrographs of cell walls from wild type (a-b) and low *e*RGL (#18) expressing (c-f) tubers immuno-labelled with LM5 (a, c-e) and LM6 (b and f).

In wild type starch parenchymal walls galactan (LM5) is restricted to the primary wall (a, arrowhead), whilst arabinan (LM6) is only absent from the middle lamella (asterisk) where it expands at corners (b). Starch parenchymal cells (sp) in transgenic tubers have considerably reduced amounts of galactan (c, d and e) and arabinan (f) in their walls (arrowhead), but vascular parenchymal cells (vp in c) retain relatively high amounts of both galactan (c) and arabinan (not shown) epitopes. Epitopes are excluded from the primary wall and localised in the expanded middle lamella (asterisk in d) and lining of intercellular spaces (e) at some cortical corners. Starch parenchymal cells contain abundant biosynthetic organelles. Scale bars a, b and e represent 2 µm, d and f 4 µm and c 10 µm; st, sieve tube.

DISCUSSION

This study reports the introduction into potatoes of an *A. aculeatus* gene that encodes a rhamnogalacturonan lyase (*e*RGL). The aerial portions of *e*RGL transformed plants appear normal, but the plants produce tubers with an altered morphology and pollen with a low fertility. The altered tuber morphology is a consequence of changes in the periderm and cortex tissues of the transgenic tubers, the result of random cell divisions and abnormal expansion in these tissues. It appears that RG I is necessary for normal periderm development, perhaps by constraining the expansion of periderm cells during development, but the exact underlying reason is not yet clear.

The isolation of CWM, followed by sugar compositional analysis showed a change in several of the constituent pectic monosaccharides (Table 1). These changes involve a reduction of both galactose and arabinose, the major constituents of RG I side-chains. Furthermore, the relative proportion of homogalacturonan was increased.

Consistent with these data, immunocytochemical studies (Figure 5 and 6) show a reduction in the abundance of galactan and arabinan epitopes (side-chains of RG I) and an altered location of the residual galactan epitopes in parenchymal cell walls. Considering that the *e*RGL can only degrade the RG I backbone (Kofod *et al*., 1994), the subsequent altered location of the galactan and arabinan can only be explained if they are not cross-linked to other wall polymers.

The GBSS promoter is activated during the process of stolon initiation followed by the tuber formation (Visser *et al.,* 1991). During this process of tuber development *e*RGL is released into the apoplast of the stolons. Once the *e*RGL is released into the wall, it may fragment the RG I present in the wall or newly synthesized and secreted RG I molecules before they are integrated into the wall. *e*RGL cleaves the RG I backbone (at alternating regions of α-L-Rha-(1,4)-α-D-GalA bearing single unit Gal side-chains attached to Rha; (Kofod *et al*., 1994; Mutter *et al*., 1998)) to give Rha-GalA fragments of DP 4-10 with Rha at the reducing end, and possibly also larger fragments. The loss and altered location of the LM6 epitope and the decreased arabinose content is less profound compared with the decrease in galactan. The monosaccharide compositional data confirm these observations. This suggests a larger decrease of RG I with galactan side-chains compared to RG I with arabinan side-chains. A possible explanation is the higher affinity of the *e*RGL for the galactose-containing RG I which is in line with the kinetic studies of Mutter *et al*. (1998).

In WT plants, the LM5 epitope is found in the primary cell wall, the LM6 epitope occurs throughout walls, but both are excluded from the expanded middle lamella at cell corners. The loss and altered location of the majority of the LM5 and LM6 epitopes suggests that the RG I has been degraded into smaller fragments which are subsequently re-distributed into the middle lamella and cell corners. This suggests that the RG I backbone is important for anchoring these side-chains within the primary wall. The galactan and arabinan side-chains are thus not likely to be cross-linked to other immobile wall molecules. This is in line with ^{13}C NMR spectroscopic studies of

onion pectin which show that galactan side-chains are highly mobile but glycosidically linked to RG I (Foster *et al.*, 1996; Ha *et al.*, 1996). The mechanism by which the fragments are subsequently re-distributed is as yet unknown, but the process of phase separation proposed by MacDougall *et al*. (1997) to be involved in cell wall assembly is a possible mechanism.

There is little effect on the distribution of LM5 and LM6 epitopes in the outer cortical walls and vascular tissue of the *e*RGL expressing tubers. Both of these tissues are characterized by a reduction in the number of intracellular starch granules, so presumably the GBSS promoter is relatively inactive here (compared to the perimedullary parenchymal cells) and hence it would be expected that the *e*RGL activity would also be reduced. It is also possible that the turnover of the RG I in these walls is greater than for other cells, or that the RG I in these cells has a higher degree of acetylation, resulting in a less efficient degradation by the *e*RGL (Kauppinen et al., 1995; Kofod et al., 1994; Schols et al., 1990a).

Further, the vascular walls of WT tubers contain more LM6 arabinan than LM5 galactan epitopes (Bush and McCann, 1999). The reduced affinity of *e*RGL for the arabinan-containing RG I (Mutter et al., 1998), could be another explanation for the relatively low reduction of LM5 and LM6 epitopes in these tissues.

Our results prove that the *e*RGL has indeed been active and able to modify the composition of the tuber cell wall, which resulted in several modifications, including a decrease in RG I, and a relative increase in homogalacturonan. The histological appearance and tuber morphology may be a result of the re-location and removal of the RG I backbone from the cell wall.

The degradation of the RG I backbone correlates to a decrease of both galactan and arabinan that are normally attached to this backbone. Different studies (as reviewed by Willats *et al.*, 2001) have shown that different forms of RG I (bearing either galactan or arabinan) may occur in a developmental fashion at specific locations in the plant suggesting that they have a specific function. The deposition and degradation of galactan has been correlated with the firmness of several plant tissues (Jones *et al*., 1997; McCartney *et al*., 2000; Redgwell *et al*., 1997). However, Sørensen *et al*. (2000) showed that in transgenic tubers expressing an endo-1,4-β-galactanase in the apoplast, the galactose content of the wall is reduced to 30% of the wild type, but this had no effect upon the histological or morphological structure of tubers. One explanation for this is that the removal of galactan by the endo-galactanase was not co-ordinated with other developmental processes in the plant, and that therefore the impact on the tuber's morphology and histology was less pronounced.

The specific removal of RG I arabinan side-chains by the expression of an apoplast-directed endo-1,5-α-arabinanase (Skjøt *et al*., 2002) resulted in transgenic potato plants with severe phenotypic changes: the plants did not produce side shoots, flowers or stolons, and were unable to produce tubers (Skjøt *et al.*, 2002). When the same endo-arabinanase was targeted to the Golgi complex, the potato plants and tubers showed no detrimental phenotype, but tuber cell walls contained significantly reduced levels of

arabinose and LM6 epitopes (Skjøt *et al.*, 2002). Furthermore, the *Arabidopsis mur4* mutant (Burget and Reiter, 1999), showing a reduction of L-arabinose to 50% of WT levels, does not show any phenotypic changes. Although arabinan side-chains are developmentally and spatially regulated structures (Bush *et al.*, 2001; Orfila and Knox, 2000; Orfila *et al.*, 2001; Willats *et al.*, 1999; Willats *et al.*, 2001) they have yet to be linked to a physiological function.

Even though RG I galactan and arabinan side-chains have an, as yet enigmatic, role to play *in planta*, the backbone must also play an important role in the integrity and function of the wall, since its degradation by *e*RGL in potato tubers results in morphological changes. This function may be to secure side-chains and possibly other polysaccharides to specific locations in the wall. Our experiments confirm that the approach of introducing fungal cell wall degrading enzymes *in muro*, can be very useful for attributing a biological significance to a particular wall polysaccharide.

EXPERIMENTAL PROCEDURES

Materials
The cDNA vector pYES2/*e*RGL, containing the *e*RGL from *A. aculeatus* (Kofod *et al.*, 1994), was a gift from S. Kauppinen (Novo Nordisk A/S, Bagsværd, Denmark). The α-amylase and pullulanase to de-starch cell walls were obtained from Boehringer (Alkmaar, The Netherlands) and Megazyme (Bray, Ireland) respectively.

Vector construction and transformation of potato plants
The granule bound starch synthase promoter was amplified by PCR from the vector pPGB121s (Visser *et al.*, 1991) with primers 5'GATTACGCCAAGCTTTAACG3' and 5'GGTTTTGTCGACGAAATCAGAAATAATTGGAGG3' introducing a *HindIII* site 5' and a *SalI* site in the 3' end of the PCR product. Subsequently the fragment was cloned into the pBI121 vector (Datla *et al.*, 1992) as a *HindIII*/*SalI* fragment. This pPGB121s-new vector was digested with the restriction enzyme *SalI*, blunt-ended with Klenow enzyme, and after heat inactivation of the two enzymes further digested with the restriction enzyme *XbaI*. The vector pYES2/*e*RGL was digested with the restriction enzyme *BamHI*, blunt ended with Klenow enzyme, and after heat-inactivation of the two enzymes further digested with the restriction enzyme *XbaI*. The treated vector and the cDNA insert were purified by agarose gel electrophoresis and ligated creating the DNA construct pPGB121s-new-*e*RGL.
In vitro shoots of the *Solanum tuberosum* cultivar Karnico were used for *Agrobacterium tumefaciens* mediated transformation (Visser *et al.*, 1989). After regeneration of *in vitro* shoots on selective kanamycin medium, the shoots were transferred to the greenhouse to generate mature plants.

Northern analysis
RNA was extracted from 1 tuber of each transgenic line as described by Kuipers *et al.* (1994). RNA gel blotting and hybridisation was performed using 40 μg of tuber RNA per sample, as described by Sambrook *et al.* (1989). The membranes were hybridized with a ^{32}P-ATP labelled 1-kb *KpnI-XbaI* fragment of pYES2/*e*RGL. A 2.3-kb *EcoRI* fragment of a potato 28S ribosomal RNA gene was used as a control (Landsmann and Uhrig, 1985).

Activity measurements

Frozen potato tissue was ground to a powder under liquid N_2 using a mortar and pestle. Approximately 1 g of the ground tissue was then homogenized using an Ultra-Turrax TP 18-10 (14,000 rotations min^{-1}, Ika Werk, Staufen, Germany) in 2 ml 0.25M sodium phosphate buffer (pH 6.5, 4°C) containing 0.4M NaCl. After 1 h at 4°C (with periodic shaking), the suspension was centrifuged (10 min, 2,000g) and the supernatant was used as a crude enzyme extract (Vincken *et al*., 1997).

A portion (25 µl) of this supernatant was added to 225 µl (or 5 and 245 µl in case of high activity) 0.25% (w/v) solution of saponified rhamnogalactorunan from apple (modified hairy regions, MHR (Schols *et al*., 1990b)) in 0.1M sodium phosphate buffer pH 5.0. The use of apple RG fractions in this experiment is supported by its well described degradation patterns and its easy extractability (in contrast to potato RG). After a 15 min, or overnight, incubation at 40°C in a head over tail-mixer, the enzymes were inactivated by heating for 5 min at 100°C. Fragmentation of the saponified apple RG was determined using HPSEC (BioGel TSK 40XL, 30XL, and 20 XL columns in series) (Mutter *et al*., 1996; Schols *et al.*, 1990a; Vincken *et al.*, 1997).

Isolation of cell wall material from potato tubers

For each isolation of cell wall material (CWM), 300 g (fresh weight) of frozen potato tuber cubes was ground to a fine powder under liquid N_2. The tissue was homogenised in a mixed-cation buffer (10mM NaOAc, 3mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, pH 6.5), containing Triton 100 (2 mg/ml), using an Ultra-turrax homogenizer. The residue was washed with mixed-cation buffer, resuspended in 50% acetone and extracted by saturation with phenol. This residue (washed with mixed-cation buffer) was cryomilled, added to mixed-cation buffer and heated for 20 min at 70°C. Starch was removed by an overnight incubation with α-amylase and pullulanase. When starch removal was complete the cell walls were filtered on a grade 3 sintered glass funnel and sequentially dried in 50, 75, 90 and 100% acetone. (The method is described in more detail as method 1A in Jardine *et al.*, 2002).

Sequential extraction of CWM of wildtype (WT) potato and transformant *e*RGL #9

Potato CWM (1.5 g) was sequentially extracted with 0.5M imidazole-HCl, pH 7 (3 times 150 ml) at room temperature for 2 h (Chelating Agent Soluble Solids, ChSS), and washed with distilled water (2 times 200 ml); the water and ChSS extracts were combined. After this, the forth-coming residue was extracted with 0.05M NaOH (3 times 150 ml) at 4°C for 2 h (Dilute Alkali Soluble Solids, DASS). Subsequently, 1M KOH containing 0.02M $NaBH_4$ (3 times 150 ml) was used as an extractant at room temperature for 2 h (1M Alkali Soluble Solids, 1M ASS). Finally, the residue was extracted with 6M KOH containing 0.02M $NaBH_4$ (3 times 150 ml) at room temperature for 2 h (6M Alkali Soluble Solids, 6M ASS), and washed with distilled water (3 times 200 ml); the water and 6M ASS extracts were combined. After each extraction step, solubilised polymers were separated from the insoluble residue by filtration over a grade 3 sintered glass funnel. When changing extractants, the funnel was rinsed in the opposite direction with the new extractant applying low air pressure. The filtrate was collected and added to the next extraction step. All extracts were acidified to pH 5.2 (if necessary) by glacial acetic acid, dialysed against distilled water, concentrated by vacuum evaporation, dialysed again, and finally freeze dried. The 6M ASS fraction was dialysed against tap-water before dialysing against distilled water. The final residue was suspended in water, acidified to pH 5.2, dialysed and freeze dried.

Degradation of the 6M ASS fraction of WT and *e*RGL #9 potato CWM

The 6M ASS fraction of both WT and *e*RGL #9 (10 mg) was suspended in 5 ml of a 0.05M NaOAc buffer pH 5 containing 0.01% NaN_3 at 80°C. Subsequently, 490 µl of this substrate solution was incubated head-over-tail at 40°C with 10 µl (0.08 mg protein/ml) of the commercially available *e*RGL (Batch PPJ 4471; Novo Nordisk, Bagsværd, Denmark). After 16 h the incubation mixtures were heated at 100°C for 15 min to inactivate the enzyme, and then centrifuged (10 min, 20,000 g). The

degradation products in the supernatant were analyzed by High-Performance Anion-Exchange Chromatography (HPAEC) at pH 12 as described by Daas *et al.* (1998). Samples were separated on a CarboPac PA-1 column preceded by a CarboPac PA-100 guard column. A 40-min linear gradient of 0.1-0.5M NaOAc in 0.1N NaOH at a flow rate of 1 ml/min was used, followed by a 5 min gradient to 1M NaOAc. After 5 min of elution with 1M NaOAc in 0.1N NaOH, the column was equilibrated with 0.1M NaOAc in 0.1N NaOH for 15 min. The compounds were detected with a PAD-detector (Electrochemical detector ED40, Dionex, Bavel, The Netherlands) in series with a UV-detector (Spectrasystem UV1000) set at a wavelength of 235 nm. A 0.25% w/v solution of saponified apple MHR was digested with fungal *e*RGL under the same conditions as the 6M ASS fractions for peak identification. To ensure that the observed degradation was not caused by the incubation conditions, enzyme blanks containing 490 µl of 6M ASS fraction and 10 µl of the inactivated enzyme were analysed.

Immunogold labelling and microscopy
Small pieces of cortex and perimedullary tissue cut from freshly harvested wild type and *e*RGL transformed tubers were fixed in 2.5% (v/v) glutaraldehyde-0.05M sodium cacodylate containing 0.05% (v/v) NP-40 (Sigma, Poole, U.K.) and then processed for low temperature L.R. White resin embedding, as described previously (Bush and McCann, 1999). Tissue sections (0.5 µm and ultra-thin) were labelled with mAbs LM5 (recognizes (1→4)-β-D-galactan, (Jones *et al.*, 1997)) and LM6 (recognizes (1→5)-α-L-arabinan, (Willats *et al.*, 1998)), followed by silver enhancement of 5 nm gold conjugates (Bush and McCann, 1999). Gold-labelled and silver-enhanced resin sections were examined by confocal laser scanning microscopy or by transmission electron microscopy as described previously (Bush and McCann, 1999).

Analytical procedures
Residual starch was determined using the Boehringer (Mannheim) enzymatic package.
Neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples (for CWM and the residue) 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. Sugar composition analysis of the various extracts was performed according to Englyst and Cummings (1984) using 2M TFA (for 3 h at 121°C) instead of H_2SO_4 for polysaccharide hydrolysis. It should be noted that during the extraction procedure some residual starch in the CWM is also extracted, notably in the 1M ASS and DASS fractions. The values for ChSS, DASS, 1M ASS and 6M ASS have been corrected for the presence of starch. For this, it was assumed that (i) all cellulose is present in the residue, (ii) the residue contains no starch, (iii) all xylosyl residues are derived from xyloglucan (only small amounts of xylan are present in potato (Ryden and Selvendran, 1990) and (iv) the Glc:Xyl ratio in potato xyloglucan is 2 (Vincken *et al.*, 1996). With this, the amount of glucose derived from starch can be estimated for the various extracts, which was subsequently subtracted from the total amount of glucose in these fractions.
Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) using an auto-analyzer (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample.

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3

**Modulation of the cellulose content of potato
(*Solanum tuberosum* L.) tuber cell walls**

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SUMMARY

Four potato cellulose synthase (*CesA*) homologs were isolated by screening a cDNA library made from developing tubers. One full-length cDNA clone was obtained and used for up- and down-regulation of the corresponding RNA expression levels resulting in respectively increased and decreased cellulose levels (ranging from 50 to 200% of the WT level) in potato tuber cell walls. Transgenic plants, in which the GBSS promoter was used to drive expression of the CesA homolog in tubers, showed normal plant and tuber development. Transformants were screened by Fourier Transform Infra-Red microspectroscopy (FTIR) which identified transformants with altered levels of cellulose in their tuber cell walls compared to wild type (WT) plants, and this was confirmed by measurements of cellulose content.

Potato transformants with decreased cellulose levels did not show any alterations to other cell wall polysaccharides. The plants with increased cellulose levels showed increased uronic acid and decreased galactan levels. This may be explained by shifts in the levels of the corresponding UDP-sugars as a result of the elevated rate in cellulose biosynthesis.

INTRODUCTION

The importance of the load-bearing cellulose-xyloglucan network for plant cells has been the subject of several studies. The studies with cellulose synthesis inhibitors in two dicotyledonous cell cultures, tomato (Shedletzkey *et al*., 1990; Wells *et al*., 1994) and tobacco (Shedletzkey *et al*., 1992), have shown that cellulose deficiency can be compensated by the presence of a pectin-rich wall. However, the discovery of the genes that encode the putative catalytic subunit of cellulose synthase (*CesA* genes) (Pear *et al*., 1996) provides an alternative strategy to modulate cellulose content by transgenic approaches. So far, this resulted mostly in the identification and characterization of *Arabidopsis CesA* mutants. A number of both primary and secondary cell wall mutants of the 10 family members in *Arabidopsis* have been identified in various screens for developmental phenotypes: *rsw1, ixr1, ixr2* and *prc1* (Arioli *et al*., 1998; Desprez *et al*., 2002; Fagard *et al*., 2000; Scheible *et al*., 2001) and respectively *irx1*, *irx3* and *irx5* (Turner *et al*., 2001). A number of these mutants show a reduction in the cellulose content of the cell walls, along with modifications to the other cell wall polysaccharides (e.g. increased hemicellulose fractions and decreased galactose levels).

Nevertheless, the identification of more and more plant *CesA* genes also enables the direct modulation of the cellulose content in crop plants by altering the expression level of these genes. However, it is necessary to study a range of transformed plants with the same construct in which cellulose content has been titrated to different amounts within the wall. This will provide information on the tolerance of plants to different levels of

cellulose in the wall, and possible feedback mechanisms that alter the proportions of non-cellulosic wall polysaccharides. A first example of this is the down-regulation of a *CesA* gene in tobacco (*Nicotiana benthamiana*) by using the virus-induced gene silencing (VIGS) system (Burton *et al*., 2000). The *CesA* gene expression was inhibited upon infection with the virus giving decreased cellulose levels (25% reduction) with a concomitant increase in unesterified homogalacturonan, resulting in a 'dwarfed' phenotype. The phenotype reverted to wild-type after about 4 months, when the plant recovered from the viral infection. The down-regulation of three *CesA* genes in *Arabidopsis* resulted in phenotypic effects similar to, but not identical to the comparable mutants (Burn *et al*., 2002). The phenotypes shown by these transgenic plants were unstable and cellulose levels were not significantly reduced.

The interest of modifying the potato cell wall composition arises from it being a major waste product from the starch industry. As described by Vincken *et al*. (1999) the possibility to produce potato pectin with a specific composition may be useful for e.g. the food industry. Based on the studies describing cellulose deficiencies in other plants a reduced cellulose content is also in potato likely to result in increased levels of pectin. So far, there is no obvious benefit of increased cellulose levels in potato tubers but exploring this possibility may be useful for final applications in other crop plants as e.g. cotton and flax. In order to determine the tolerance of potato tubers to modifications in cellulose content in the cell wall, a full length potato *CesA* gene was isolated and used for sense and antisense expression. Various transgenic lines were obtained showing both increased, as well as decreased, levels of cellulose in comparison to wild type plants.

RESULTS

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, see figure 1a) (Pear *et al*., 1996) of a potato *CesA* cDNA. A 500-bp fragment, showing high homology with previously identified *CesA* genes, was amplified and used for the screening of a cDNA library made from swelling stolon tips. Four different potato *CesA* clones were isolated, of which *StCesA14* was full length. The other three lacked up to 1 kb of the 5' region, and were named *StCesA7*, *10* and *17*.

Sequence comparison of the isolated cDNA clones
As three of the four potato *CesA* clones were not full length, sequence comparisons were based on the class-specific region (CSR) of the cDNA clones. This region is important in revealing sequence divergence and for the clustering of different *CesA* genes from different plant species (Vergara and Carpita, 2001). Figure 1b shows an amino acid sequence alignment of the CSR regions of the potato *CesA* clones. The four

sequences share only 57% sequence identity on average. *StCesA7* and *StCesA17* are genetically more closely related to each other (86% identity) than to *StCesA10* and *StCesA14*.
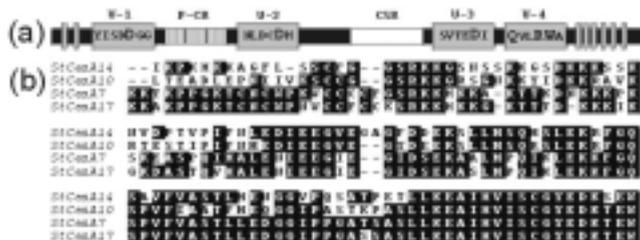

FIGURE 1.
a, Schematic representation of the plant *CesA* genes. Grey shaded boxes represent the 4 conserved 'U-motifs' containing the D,D,D,QxxRW motif. The grey bars represent the 8 putative transmembrane regions. The Plant Conserved Region (P-CR) is shown as a striped box, and the Class Specific Region (CSR) is shown as a white box.
b, Sequence alignment showing the amino acid sequences of the CSR region (HVR, Pear *et al*. 1996) for the four potato *CesA* sequences; AY221086 (*StCesA7*), AY221089 (*StCesA10*), AY221087 (*StCesA14*), AY221088 (*StCesA17*). Blocked residues represent amino acids that are identical for at least two sequences. Analyses were performed using the Clustal analysis option of the DNA-Star program package with gap penalties and gap-length penalties of 10 and a PAM250 weight table.

Modification of CesA RNA expression in potato plants
Several constructs were made to explore the possibility of modulating cellulose biosynthesis during plant, and specifically tuber, development in potato. The full-length *StCesA14* cDNA sequence was cloned in both sense (SE) and anti-sense (AS) orientation 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-*CesA* transformants developed normally and did not show any visual abnormalities (including the tubers) in comparison to wild-type plants.

Transformation with a construct having the antisense *StCesA14* 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. The CaMV promoter is expressed in most plant tissues in multiple stages of development and gene expression. The few plants that were transformed with the antisense CaMV construct did not show any morphological abnormality in comparison to WT. Since Fourier Transform Infra Red microspectroscopy (FTIR) did

not show any cell wall modifications, these plants most likely have undetectable changes to cellulose due to very low RNA inhibition efficiency. The difficulty in obtaining transformants with the constitutive CaMV 35S promoter thus indicates the importance of choosing the right promoter for modulating cellulose levels in the plant. These plants were not studied further.

Initial screening of potato transformants by FTIR Spectroscopy

FTIR microspectroscopy was used to screen the transformants for alterations in their cellulose content and/or cell wall composition. Chen *et al*. (1998) have described FTIR as a screening method to identify cell wall mutants. A problem in our analysis was the high starch content (about 90% DW) in potato tubers. Starch and cellulose are both crystalline polysaccharides that give rise to absorbances in the carbohydrate fingerprint region of the IR spectrum. Consequently the high starch content can mask changes to cellulose content in the transformants. It was not possible to discriminate between spectra obtained from sections of transgenic and control plants by Principal Component Analysis (PCA) . Therefore, the sections were incubated for 15 min at 90°C to gelatinize the starch. The crystalline cellulose is not affected by this treatment. FTIR spectra were collected from a series of control plants before and after the heat treatment. After assigning these treatments as two different groups, exploratory PCA showed group separation (Figure 2a). By using 4 PC scores to calculate Mahalanobis distances (Kemsley, 1998), 85% of the spectra were assigned to their correct group. The use of 9 PC scores resulted in correct assignment of 99% of the spectra. Both PC loadings 4 and 9 showed peaks characteristic of starch (Wilson and Belton, 1988; negative peaks at 850, 930, 1026, 1082, 1110 and 1155 cm^{-1}, Figure 2b), which confirms that the crystalline starch signals can be removed in this way.
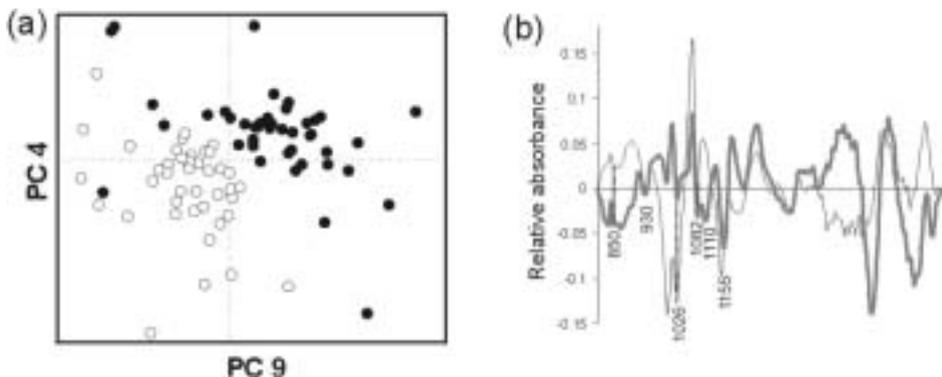
FIGURE 2.
Exploratory PCA of infrared spectra obtained from heat-treated and untreated potato tuber tissues from control plants.
a, Exploratory PCA discriminates tissues treated at 90°C (open circles) from the untreated tissues (closed circles), using the 4th and 9th PC scores. **b**, The corresponding loadings for PC4 (thick grey line) and PC9 (thin black line) show features characteristic of starch (850, 930, 1026, 1082, 1110 and 1155 cm^{-1}).

Treatment of both wild-type and transformed tuber sections for 15 min at 90°C followed by FTIR and exploratory PCA, discriminated several transgenics from wild-type plants, as outliers from the wild-type cluster (data not shown). A number of SE-*CesA* and AS-*CesA* transformants clearly separated from WT. To improve discrimination of the SE-*CesA* transformants, these were assigned as two groups representing plants with increased and decreased levels of cellulose, for comparison with WT.
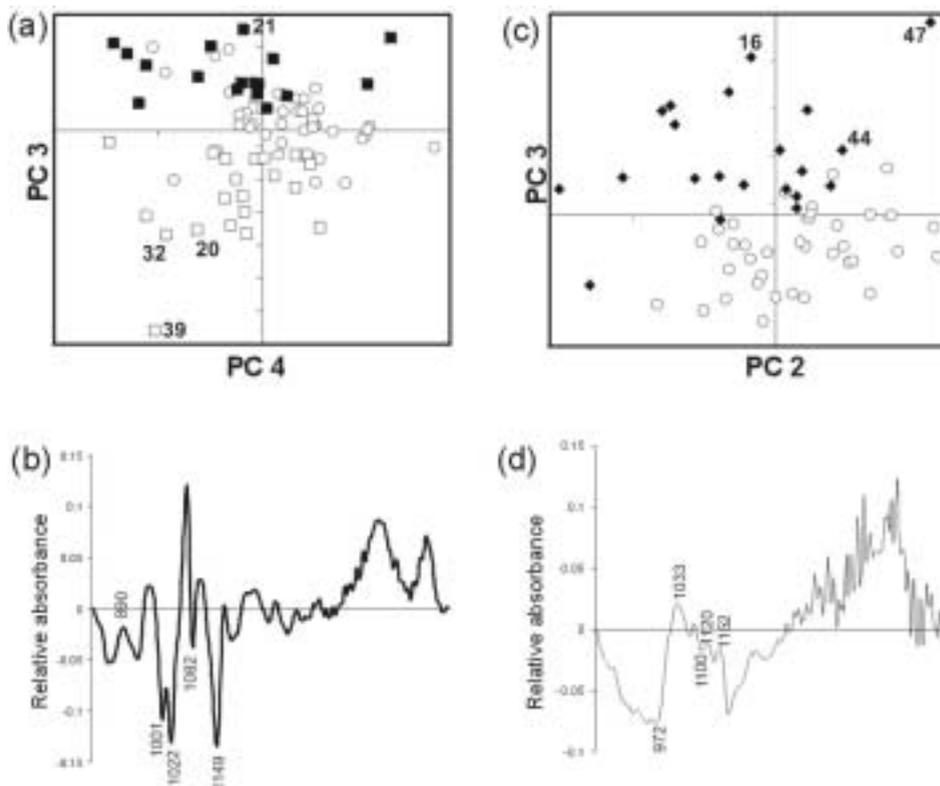
FIGURE 3.
Exploratory PCA of infrared spectra obtained from transformed and control plants.
a, Exploratory PCA discriminates the SE-*CesA* transformants (closed and open squares, respectively referring to plants with decreased and increased levels of cellulose) from the control plants (open circles) by using 3 PC scores. **b**, PC loading 3 shows features characteristic for pectin (890, 1001, 1022, 1082 and 1149 cm^{-1}). SE-*CesA* transformants #20, 21, 32 and 39, were selected for further analysis. **c**, Exploratory PCA discriminates the AS-*CesA* transformants (closed diamonds) form the control plants (open circles) by using 3 PC scores. **d**, PC loading 3 shows features characteristic for cellulose (1033 and 1120 cm^{-1}) and pectin (972, 1100 and 1152 cm^{-1}). AS-*CesA* transformants #16, 44 and 47 were selected for further analysis.

Figure 3a shows the cluster plot of the two groups of SE-*CesA* transformants with control plants. Calculation of Mahalanobis distances using 3 PC scores results in correct group assignments of 57 of the 81 (70%) plants. PC loading 3 (Figure 3b) shows characteristic features of pectin (Séné *et 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. Nevertheless, previous experiments have shown that reduced cellulose levels are often accompanied by modifications in pectin levels and structure (Burton *et al*., 2000; His *et al*., 2001; Shedletzky *et al*., 1992; Wells *et al*., 1994). Therefore, based on this PCA plot, 4 SE-*CesA* transformants were selected for further analysis. Transformant #21 was selected as potentially cellulose-deficient and transformants #20, 32 and 39 for potentially higher levels of cellulose compared with WT.

Similarly, AS-*CesA* lines could be discriminated from the control-lines by PCA. Figure 3c shows a cluster plot with group separation largely by PC3: calculation of Mahalanobis distances using 3 PC scores results in 66% correct assignment to the two groups. PC loading 3 (Figure 3d) shows features characteristic for cellulose (Liang and Marchessault, 1959; +1033 and +1120 cm^{-1}) and pectin (-972, -1100 and +1152 cm^{-1}). Based on the cluster plot, the AS-*CesA* transformants #47, 16, and 44, were selected for further analysis, predicting #47 and 16 to be most distant from the WT.

StCesA14 expression levels and FTIR results are correlated

RNA was isolated from the selected transformants. RT-PCR was performed with different primer combinations to determine selectively the expression level of the four potato *CesA* genes and the *StUGE51* gene (UDP-Glc-4-epimerase from *Solanum tuberosum*, Chapter 5), which is used as a control because of its constitutive expression. Figure 4 shows the RT-PCR results for both the SE-*CesA* and the AS-*CesA* transformants with the *StCesA14* (a) and *StUGE51* (b) primers. Comparison of the amount of product amplified with the *StUGE51* primers and the *StCesA14* primers clearly shows a variation of the *StCesA14* expression in the transformants in relation to the WT plant. *StCesA14* RNA expression is clearly lower in SE-*CesA*-21 and higher in SE-*CesA*-39. For the other transformants this is difficult to determine. PCR amplification with the other potato *CesA* primer combinations showed no variation in the expression levels of these three genes in comparison to each other and the WT plant (not shown).

FIGURE 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 *StCesA14* **(a)** and *StUGE51* **(b)**.
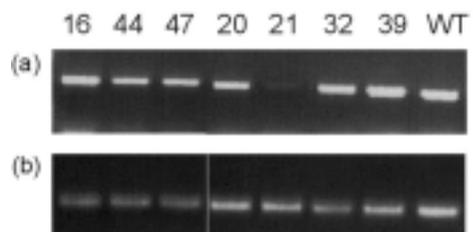
Modified cellulose content in the transformants

Tissue sections (90 µm) of the selected sense and antisense transformants were imaged by bright-field microscopy, and by fluorescence microscopy after staining cellulose in cell walls with Calcofluor White. Calcofluor White images of the cortex and perimedullary regions from the transgenic plants in comparison with WT (data not shown) did not show any difference in staining intensity. The cells and tissue anatomy appeared normal.

Cell wall material (CWM) was isolated from the potato tubers and cellulose content determined by a colorimetric assay, and expressed as percentage (w/w) of cellulose per total 'crude' CWM (crude means, without removal of the starch by a heat and enzyme treatment). Considering that of this 'crude' CWM about 90% will be starch, cellulose levels will be around 3-4 % in stead of the 30-40% usually found in CWM (Ryden and Selvendran, 1990).

FIGURE 5.
Percentage of cellulose in the transformed and control plants.
The cell wall material 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 CWM. Measurements were performed in duplicate and the bars represent the standard deviation.
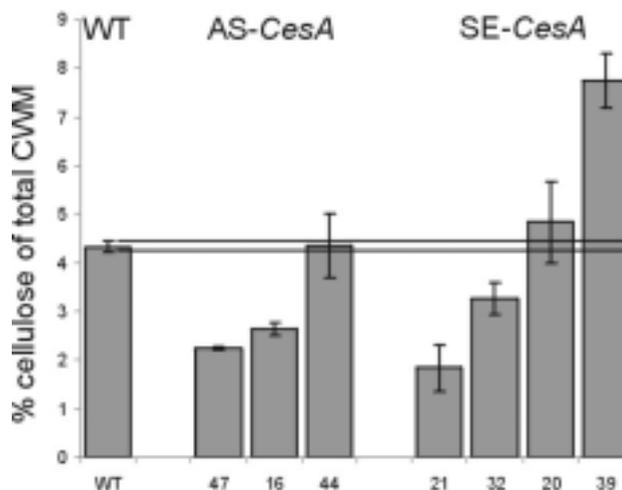
Figure 5 shows the variation in cellulose content of a number of transformants and a WT control. Even though the cellulose levels determined by this method appear very low, the standard deviations represented in figure 5 show the reliability of the method. The level of cellulose varies among the different transgenic plants of which some show a very low percentage (down to 2% for AS-*CesA* 47 and SE-*CesA* 21) of cellulose in comparison to the WT (4%). Both transformants SE-*CesA* 20 and 39 (5 and 8% respectively) have increased cellulose content in comparison to the WT, while SE-*CesA* 21 and 32 show a decrease. The values for cellulose content are consistent with the results of the FTIR/PCA analysis. Line 44 of the AS-*CesA* plants has WT levels of cellulose while 47 and 16 have reduced cellulose content.

Modified monosaccharide composition in the transformants

Table 1 shows the monosaccharide composition of the CWM isolated from potato tubers of the transformants and control plants. A starch degradation step was included in the preparation of CWM to reduce the amount of glucose such that baseline separation with galactose and inositol (the flanking peaks) could be obtained by gas chromatography. As the samples are still contaminated with starch-derived glucose, the amount of glucose was set to zero for the calculation of the relative mol% shown in Table 1. The SE-*CesA* transformants 20 and 39, which have high levels of cellulose, show a significant decrease in galactose and increase in uronic acid. The AS-*CesA* plants (16 and 47), in which decreases of cellulose are observed, vary little from WT.

TABLE 1. Monosaccharide composition (mol %) of cell wall material isolated from tubers of wild type and *StCesA14* sense and antisense expressing potato plants. The values represent the average of a duplicate analysis 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
AS-*CesA* 47	3 ± 0.3	$13 \pm 0.6^*$	6 ± 0.2	0^*	37 ± 1.4	40 ± 1.5
AS-*CesA* 16	4 ± 1.5	10 ± 0.3	$4 \pm 1.5^*$	0^*	38 ± 1.1	43 ± 2.1
AS-*CesA* 44	3 ± 0.2	$13 \pm 0.5^*$	$8 \pm 0.6^*$	$3 \pm 0.4^*$	34 ± 0.1	39 ± 1.2
SE-*CesA* 21	2 ± 0.4	12 ± 0.4	5 ± 0.1	2 ± 0.1	$35 \pm 1.6^*$	45 ± 2.7
SE-*CesA* 32	2 ± 0.1	11 ± 0.1	6 ± 0.5	2 ± 0.1	38 ± 0.5	40 ± 0.8
SE-*CesA* 20	2 ± 0.6	13 ± 0.0	7 ± 0.1	2 ± 0.1	$27 \pm 0.6^*$	$49 \pm 0.9^*$
SE-*CesA* 39	2 ± 0.4	11 ± 0.8	$8 \pm 0.5^*$	2 ± 0.3	$25 \pm 0.2^*$	$51 \pm 0.5^*$
WT	2 ± 0.3	11 ± 0.2	6 ± 0.1	2 ± 0.2	38 ± 1.7	42 ± 2.7

*Values showing significant alteration in comparison with the WT values.

DISCUSSION

To establish the boundaries for up and down-regulation of cellulose content in potato tubers, transgenic plants were generated with decreased and increased *StCesA14* RNA expression levels. FTIR microspectroscopy in combination with exploratory PCA as described by Chen *et al.* (1998) was successfully used for the identification of interesting potato transformants among the AS- and SE-*CesA* transformants.

A comparison of a group of WT plants with a group of mutant plants which all contain the same mutation, will show a good separation of the two groups (Chen *et al.*, 1998). In our transformants, the genetic modification is expected to induce different levels of effect in the various transgenic lines, due to the introduction of multiple copies of the transgene and variation in the level of transcription. The plants that only show a minor effect should cluster with the control plants, whereas the other transformants should be outliers from the WT cluster proportional to the effect. This is exactly what we observed with FTIR/PCA, and this was confirmed by cellulose analysis of isolated CWM. The transformations thus clearly resulted in the generation of a range of transgenics with varying cellulose content. RT-PCR analysis confirmed modified *StCesA14* expression levels of the selected transformants.

The generation of transformants has several advantages in comparison with the mutant analysis approach. First, the antisense approach enables the direct down regulation of multiple genes from one gene family. This can of course also be a disadvantage if one wants to specifically modulate the expression of a particular gene. Second, most *CesA* mutants so far show mutations which result in disabled, but still partly functional, *CesA* proteins. This may be an explanation for the variation in phenotype of the *rsw1-1* mutant and the *CesA1* antisense plants (Burn *et al.,* 2002). A complete knock out of a particular protein requires mutations at specific sites which abolish the function of the protein completely. The antisense approach directly results in a reduced amount or absence of a particular protein. Even when a 100% knock out is lethal, plants can be generated which show reduced levels of the protein. In this way, transformants can be obtained with a range of the phenotype of interest. The combined use of both antisense as well as sense expression of the *StCesA14* in our case resulted in cellulose levels varying from 50 to 200% of the WT amount.

The two plants (SE-Cesa20 and 39) with increased cellulose contents (one of which shows a doubled amount of cellulose) are with the first plant materials in which increased cellulose levels have been described. Several authors have suggested, or proven, the participation of several different *CesA* proteins in one cellulose synthase complex (Desprez *et al*., 2002; Fagard *et al*., 2000; Taylor *et al*., 2000). These ideas impose that even though the deficiency of one *CesA* gene can result in reduced cellulose levels, an increased amount of cellulose will most likely require the increased expression of several *CesA* genes participating in the same complex. The two

transformants SE-*CesA* 20 and 39 have shown differently, both have increased levels of cellulose upon increased expression levels of only the *StCesA14*. Different hypothesis can be put forward to explain this discrepancy. The fact that one cellulose synthase complex can be composed of different *CesA* subunits does not exclude the existence of complexes which are composed of only one type of *CesA* proteins. These complexes may exist in a natural situation or can be a specific effect of the increased *StCesA14* expression level in these transformants. Another explanation may find its origin in the *StCesA14* being a limiting factor in the formation of the cellulose synthase complex which it is part of. An increase of only this component will subsequently result in elevated levels of cellulose biosynthesis. This hypothesis could be further studied by generating transgenic plants with increased expression levels of different *CesA* genes on their own but also combined in one plant.

Several experiments in different plants and cell systems showed cellulose deficiency to be correlated with additional modifications in other cell wall polysaccharides (Burton *et al*., 2000; Fagard *et al*., 2000; His *et al*., 2001; Shedletzky *et al*., 1990 and 1992). In most of these experiments, the reduction in cellulose was correlated with an increase in homogalacturonan, which is often combined with a decrease in galactan. These pectic changes were suggested to be required for the formation of a stronger pectic network to compensate for the loss of the load-bearing cellulose. The higher homogalacturonan content increases the potential of forming a Ca^{2+} cross-linked pectin network, which may be facilitated by a decrease in rhamnogalacturonan-I galactan side chains.

Even though initial FTIR analysis also indicated changes in pectin composition monosaccharide analysis of the transformants showed no significant changes in potato tubers cell walls with a decreased cellulose content. The lowest amount of cellulose obtained was in transformant SE-*CesA* #21, having a 50% reduction of the WT content. Even though this is not as low as the ~95% reduction found for the DCB adapted cell lines of tomato and tobacco (Shedletzky *et al*., 1990 and 1992), it is lower than the 25% reduction in the virus-induced silencing of tobacco plants, which also showed alterations in pectic content (Burton *et al*., 2000). One explanation for this apparent inconsistency is that the reduction in cellulose has a different effect in different plants or even plant tissues or systems with different threshold limits for cellulose content before compensatory mechanisms are induced. It should be noted that in the transformed potato plants the modification was limited to the tubers. A severe effect on plant development by a complete reduction of cellulose biosynthesis may be inferred from the difficulty of generating antisense *CesA* potato plants with the constitutive CaMV promoter.

The two plants with increased cellulose contents also show an increase in uronic acid, a decrease in galactose and a small increase in xylose. A possible explanation is that the higher rate of cellulose biosynthesis results in a reduction of the UDP-Glc pool size. Due to the central position of the UDP-Glc in the biosynthetic pathway of other cell wall related UDP-sugars (Figure 6) a severe modification in its pool size is likely to affect the pool size of other nucleotide sugars as well. The UDP-Glc pool is directly linked to that of UDP-Gal and UDP-GlcA and more indirectly to other nucleotide sugars. The analysis of the cell wall monosaccharides showed a decrease for only galactose, indicating that the other nucleotide sugars are probably less affected by changes in the UDP-Glc pool size. One explanation for this could be that the UDP-Glc-4-epimerase (UGE), responsible for the conversion of UDP-Glc and UDP-Gal (Maitra and Ankel, 1971), has a high Km value (low affinity) for UDP-Glc while the UDP-Glc dehydrogenase (UGD) has a low Km value for this substrate. The UGE may thus be the first enzyme to suffer from a decrease in UDP-Glc pool size. Interestingly, we found a related effect in potato plants with increased expression levels of the UGE. These plants showed that, as a consequence of sense expression of UGE, the amount of cell wall bound galactan increased in correlation with a decrease in uronic acid (Chapter 5). This increased level of UGE results in a stronger use of the UDP-Glc pool which subsequently overrules the high substrate activity of the UGD resulting in a reduction of the uronic acid level.

FIGURE 6.
Schematic representation of UDP-sugar conversion pathways, illustrating the central position of UDP-Glc.
Abbreviations: CesA; cellulose synthase catalytic subunit, GAE; UDP-D-glucuronate 4-epimerase, Susy; sucrose synthase, SUD; soluble UDP-D-glucuronate decarboxylase, UGD; UDP-D-glucose dehydrogenase, UGE; UDP-D-glucose 4-epimerase, and UXE; UDP-D-Xylose 4-epimerase.
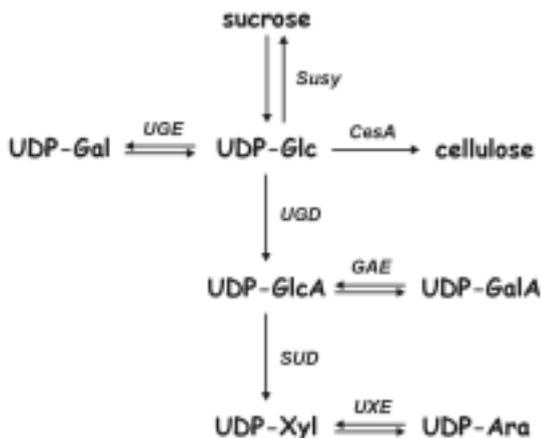
The cellulose levels in the transformed plants clearly demonstrate that it is possible to down- (to 50%) and up- (to 200%) regulate the cellulose content by modifying RNA expression levels. In our study we have used a full-length *CesA* sequence. However, it is uncertain if the expression of these sequences has affected only the individual *CesA14* gene or if it has affected other potato *CesA* genes active during tuber development.

If the plants generated in this study will indeed be useful for e.g. industrial applications will need further study. The plants with decreased levels of cellulose have not shown the expected increase in pectin components. Nevertheless, the plants with increased levels of cellulose have proven the possibility to enhance cellulose levels by increasing the expression of a single *CesA* gene, which may find an application in other crop plants.

EXPERIMENTAL PROCEDURES

Development of the primers

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; Wong *et al*., 1990; Saxena *et al*., 1990; Saxena *et al*., 1995.

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'.

Amplification of the potato *CesA* fragment

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 30s, annealing at 51°C for 30s, and amplification at 72°C for 30s. 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.

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 2x, 1x and 0.5x SSPE, 0.1% SDS at 60°C, each 2 x 15 min.

The screening yielded 11 positive clones, of which the phagemids were excised using the ExAssist Interference-Resistant 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.

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.

Vector construction and transformation of potato plants

One sense and one antisense construct was made using the full length *STCesA*14 cDNA sequence and the granule-bound starch synthase (GBSS) promoter (Visser *et al*., 1991). 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*14 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 (Benfey and Chua, 1990) 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. The constructs were checked for sense or antisense orientation of the *STCesA14* cDNA by restriction analysis and 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, the shoots were transferred to the greenhouse to generate mature plants.

Fourier Transform Infra Red microspectroscopy

Vibratome sections (70 µm) from all freshly harvested wild-type and transgenic tubers 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 µm^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 1,800-800 cm^{-1} was carried out by using WIN-DISCRIM software (E.K. Kemsley, Institute of Food Research, Norwich, U.K.).

RT-PCR method

Tubers of two or three transformed plants (one of each) were pooled and ground to a fine powder in liquid nitrogen. RNA was extracted as described by Kuipers *et al*. (1994). 50 µg of total RNA was treated with DNAseI and purified using the GenElute^{TM} Mammalian Total RNA Kit (Sigma, Zwijndrecht, The Netherlands). 9.5µg of RNA was used for cDNA synthesis in a mix containing, 100 ng primer polydT (5'-tttttttttttttttttttttttttt-3'), 1µl 25mM dNTPs, 2µl 100mM DTT, 10µl 5X First Strand Buffer and 1µl Reverse transcriptase/ Superscript^{TM} II (Life Technologies, Breda, The Netherlands) in a final volume of 50µl for 1h at 42°C. 5µl of cDNA was used in a standard PCR reaction with the following primer/Tm/cycle-number combinations. *StUGE51* primers, F(5'-ttggtaaccccaaggatttt-3'), R(5'-tttagcggacggtcatgttgttgc-3') Tm=55°C, 28 cycles. *StCesA14* primers, F(5'-gatggaaatgagttgccacgacta-3'), R(5'-ccacttcaaccgtccattgtatcc-3') Tm=58.5°C, 22 cycles. *StCesA7* primers, F(5'-ggccgaaatggtgttgctgttgt-3'), R(5'-cctatcgggcatgcagtagacg-3') Tm=67°C, 23 cycles. *StCesA10* primers, F(5'-gtgttgttttaacaggcaggctctatac-3'), R(5'-aggcttagtggatgcgggaatg-3') Tm=58.3°C, 23 cycles. *StCesA17* primers, F(5'- ttcttttttatcggtggtcgttttcc -3'), R(5'-tgcgggtgtcagcggtcatc -3') Tm=51°C, 31 cycles.

Isolation of cell wall material from potato tubers

For the isolation of CWM, one tuber from each of two or three transformed plants 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 3h at RT with continuous shaking. The CWM was pelleted by centrifugation at 16000 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.

Analysis of the cellulose content

50 mg 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.

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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Selective RNA inhibition of three potato (*Solanum tuberosum* L.) CesA genes results in reduced cellulose content and altered matrix polysaccharide composition in tuber cell walls

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SUMMARY

The class-specific regions (CSR) of four potato cellulose synthase genes (*CesA7*, *10*, *14* and *17*) expressed in potato tubers were used for specific down-regulation of the corresponding *CesA* genes. The transformants (referred to as csr7, 10, 14 and 17) showed no 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). This indicated that lines expressing constructs csr7, csr10 and csr17 had altered cell wall compositions, whereas the lines expressing csr14 did not, relative to wild-type. These results were confirmed by determination of the cellulose content and monosaccharide composition.

Csr10 and csr17 antisense lines showed reductions in cellulose content down to 50% of the WT level, as well as increased uronic acid and decreased galactose levels. Antisense *csr7* lines showed smaller decreases in cellulose content down to 75% of WT levels. The csr14 lines showed no difference in cellulose content, or other cell wall polysaccharides relative to WT. Antisense constructs based on only the CSR are specific and sufficient to down-regulate cellulose biosynthesis. Thus, different CesA proteins are likely to be co-ordinately involved in cellulose biosynthesis in tubers.

INTRODUCTION

The identification of the first plant *CesA* gene by Pear *et al.* (1996) permitted the characterization of a large multigene family. Comparison of plant and bacterial *CesA* sequences showed that, apart from the existence of highly conserved domains, the plant proteins have additional plant-specific regions (Delmer, 1999). In addition to the *CesA* gene family (e.g. 10 family members in *Arabidopsis thaliana*, http://cellwall.stanford.edu), families of similar sequences, called cellulose synthase like (*Csl*) genes, were identified. The predicted structures of the CesA and Csl proteins share the highly-conserved DDDQxxRW motif and the presence of two N-terminal and six C-terminal transmembrane domains. Nevertheless, large differences exist at the amino acid level (Richmond and Somerville, 2001; Saxena and Brown, 2000). It has been suggested that each class of *Csl* genes encodes isozymes of other, non-cellulosic, polysaccharide synthases.

Many studies are 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). An important feature revealed by mutant analysis is that different CesA proteins are required for synthesis of primary and

secondary cell wall cellulose. The three mutants *irx1* (*AtCesA8*, Taylor *et al.*, 2000), *irx3* (*AtcesA7*, Taylor *et al.*, 1999) and *irx5* (*AtCesA4*, Taylor and Turner, 2001) were isolated on the basis of an irregular xylem phenotype caused by cellulose deficiency in the secondary walls. Other mutants, *rsw1* (*AtCesA1*, Arioli *et al.*, 1998), *ixr1* (*AtCesA3*, Scheible *et al*., 2001) and *ixr2/prc1* (*AtCesA6*, Desprez *et al*., 2002; Fagard *et al.,* 2000) are cellulose-deficient in primary-walled cells.

The dramatic reductions in cellulose content in each of these mutants may be explained by the idea that different *CesA* proteins can belong to the same cellulose synthesising complex (Desprez *et al*., 2002; Fagard *et al*., 2000; Taylor *et al*., 2000;). Taylor *et al*. (2000) demonstrated a physical interaction between *irx1* and *irx3*. A phylogenetic comparison of different *CesA* genes showed that these two *CesA* genes group in different clusters of a dendrogram (Vergara and Carpita, 2001). Vergara and Carpita (2001) suggested that the region of the CesA sequences originally described as a hypervariable region (Delmer 1999) could be more accurately described as a class-specific region (CSR), revealing different sub-classes within the *CesA* gene family, that may have subtly different functions.

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 the 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 already been shown in tobacco (Burton *et al*., 2000) and potato (Chapter 3). Even though the experiments in potato clearly showed the possibility of generating plants with potato tubers having both increased as well as decreased cellulose levels it was difficult to determine if this was the result of down regulating the expression of only one specific or multiple *CesA* genes. Therefore, we used the class specific region (CSR) of four potato *CesA* genes in antisense constructs to specifically reduce the expression levels of individual *CesA* genes.

RESULTS

Relation of the potato CesA clones to other plant CesAs

Four different CesA clones were isolated from a potato cDNA library and sequenced, as described in Chapter 3. The derived amino acid sequences of the CSRs were compared with sequences from *Arabidopsis*, cotton, maize and poplar. Even though sequence homology may not be a reliable basis for prediction of function, it is a useful tool to obtain additional information when only sequence information is available. The four potato *CesA* proteins group in three different clusters of the dendrogram (Figure

1). The seven *CesA* sequences *AtCesA4/7/8*, *GhCesA1/2* and *PtCesA2/3*, which group in two other clusters, have all been suggested to be involved in secondary cell wall synthesis (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 involved in primary cell wall cellulose synthesis. We therefore predict that the four potato *CesA* genes are also involved in primary cell wall cellulose biosynthesis. The average sequence homology within one cluster containing genes from different plant species reaches up to 81% (minimum is 65%) sequence identity. This is in contrast to the sequence identity level between *CesA* genes from a different cluster but within the same plant species (e.g. 50% for *StCesA10* and *StCesA17*, which are most distantly related).
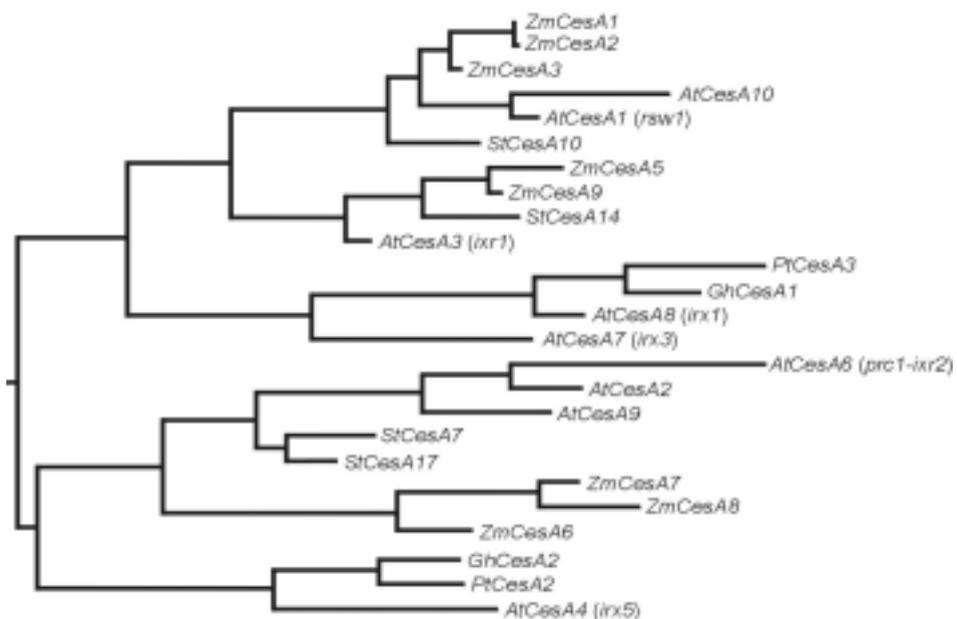
FIGURE 1.
Dendrogram representing the relationship between derived amino acid sequences of the class-specific regions (CSRs) among *CesA*s from Arabidopsis, cotton, poplar, maize and the four potato sequences. The following sequences were used: *Arabidopsis thaliana*; AF027172 (*AtCesA1*, *rsw1*), AF027173 (*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 *(StCesA7)*, AY221089 (*StCesA10*), AY221087 (*StCesA14*), AY221088 (*StCesA17*). 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.

CSR antisense constructs do not induce overt developmental phenotypes

Four constructs were made to specifically modulate the RNA expression levels of the different potato *CesA* genes. The CSR was amplified by PCR and cloned in antisense orientation 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). These constructs, and their corresponding transformants, are referred to as csr7, 10, 14 and 17, where the numbers correspond with the *CesA* clone. All constructs showed similar transformation efficiencies, comparable to a control transformation with an empty vector. For each construct, 20-50 transformed plants were generated. None of the transformants showed any visible defects during normal growth and development in comparison with the WT plants. Tissue sections from tuber material were examined using a light microscope and appeared similar to WT. Examination of tissue sections after staining with Calcofluor White (which stains cellulose) could not identify changes in cellulose levels in the transformants.

FTIR Spectroscopy is used for rapid identification of interesting transformants

For each transgenic line one tuber per plant was analyzed by FTIR spectroscopy and exploratory PCA. Preparation of de-starched sections was as described previously (Chapter 3). The plants transformed with the csr14 construct could not be discriminated from the WT plants by PCA. Mahalanobis distance was used as a means of assigning each individual spectrum to one of two groups, WT or transformant, based on distance from the mean of each group (Kemsley, 1998). Even when using up to 9 PCs to calculate Mahalanobis distance, the plants could not be correctly assigned as transformant or WT. Figure 2a shows the PCA plot based on PC loadings 1 and 2 (Figure 2b). We conclude that there is little or no difference between cell walls of WT and the csr14 lines.

For the other constructs (csr10, csr17, and csr7) the identification of transformants with a modified cell wall composition was successful. Figure 2c shows discrimination of csr10 from the control plants mostly using PC3: calculation of Mahalanobis distances using three PC scores results in correct assignment of 48 of the 49 plants. 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 WT cluster, and so these transformants were selected for further analysis. The corresponding loading for PC3 shows (Figure 2d) characteristic features for cellulose (Liang and Marchessault, 1959; 1059 nm^{-1}) and pectin (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 suggest a decrease in cellulose. Exploratory PCA shows that the csr17 transformants separated from the control plants largely by PC3 (Figure 2e): calculation of Mahalanobis distances using 3 PC scores results in correct assignment of 46 out of 48 plants. Again differences in pectin (+891, +972, -1022, -1082 and -1144 nm^{-1}) and cellulose (+1059 nm^{-1}) characteristics were found (Figure 2f).
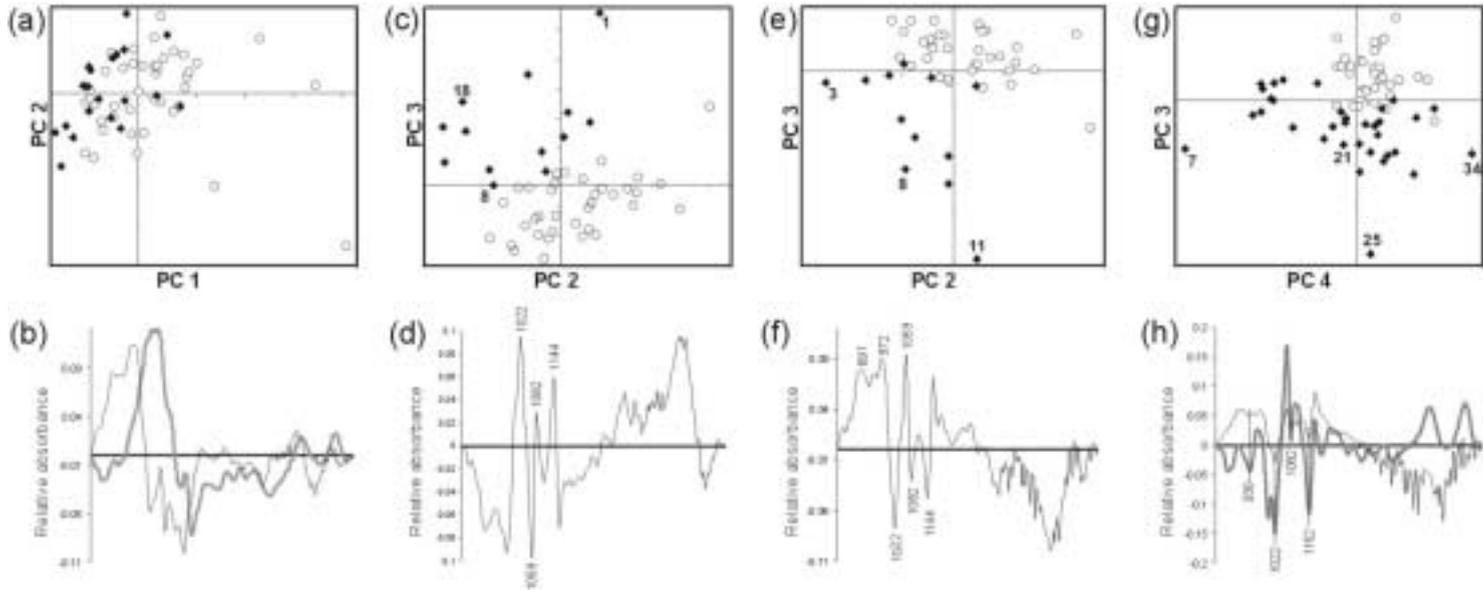
FIGURE 2.

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 x and y axis of a, c, e and f. Individual plants selected for further analysis are indicated by number.

a, *StCesA14* 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 *StCesA10* 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 *StCesA17* transformants and WT plants are separated by exploratory PCA using 3 PC scores. **f**, PC loading 3 shows features characteristic for pectin. Pectin peaks (891 and 972 nm^{-1}, PC2) as well as pectin negative peaks (1022, 1082 and 1144 nm^{-1}, PC2 and PC3) are identified. **g,** The *StCesA7* 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, 1082 and 1152 nm^{-1}) and cellulose (930 nm^{-1}).

The loading suggests an increase in pectin (mostly negative peaks) and an increase for cellulose (positive peak) for most transformants which score negative for this PC loading. 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 csr7 transformants can be discriminated from WT using a combination of PC 3 and 4 (Figure 2g): calculation of Mahalanobis distances using 3 PC scores results in correct assignment of 59 out of 70, and using 4 PC scores, 64 out of 70. Both PC loading 3 and 4 show (Figure 2h) features characteristic for cellulose (930 nm^{-1}) and pectin (1022, 1082 and 1152 nm^{-1}). Loading 4 is very similar to both of the PC 3 loadings needed to discriminate csr10 and csr17 from WT. Four csr7 transformants (#7, 21, 25 and 34), among which we predict that lines 7 and 25 will have more extreme phenotypes than lines 21 and 34, were selected for further analysis.

RT-PCR analysis confirms CesA down regulation as indicated by FTIR

For the selected transformants RNA was isolated and used for RT-PCR. Five different primer combinations were used to determine selectively the expression level of the four potato *CesA* genes and the *StUGE51* gene (UDP-Glc-4-epimerase from *Solanum tuberosum*, Chapter 5), which is used as a control because of its constitutive expression. Among the three csr10 transformants (Figure 3a), number 1 shows the most obvious decrease in *StCesA10* expression, for transformants 8 and 18 the decrease is less obvious. Even though, all three csr17 transformants (Figure 3b) seem to have less *StCesA17* RNA expressed than wild type it is difficult to distinguish between these transformants. For the csr7 transformants (Figure 3c) only transformant number 21 seems to show a decrease in expression in comparison to the WT. For the two csr14 transformants no decrease in *StCesA14* expression was detected (Figure 3d). The four *StCesA* specific primer pairs, which were used to detect altered expression induced by a particular csr antisense construct, were also used in the other csr transformants and showed that the modifications only concerned the gene at which down-regulation was aimed (data not shown).

FIGURE 3.
RT-PCR analysis of the selected transformants and wild type tuber RNA.
Panel a, b, c and d shows the PCR products visualised with EtBr after agarose gel electrophoresis for the csr10, csr17, csr7 and csr14 transformants respectively. The upper panels show the product amplified using the csr-specific primers while the lower panels show the product amplified using the *StUGE51* primer pair. Amplification of the *StUGE51* was used as a control because of its constitutive expression.
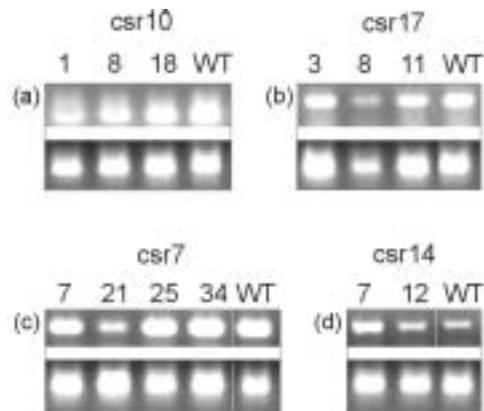
Transformants show a reduced cellulose content

A colorimetric assay was used to determine the cellulose content in the cell walls of the various potato tubers. The cell wall material (CWM) used for this analysis still contains about 90% starch. Therefore cellulose levels determined in this assay are around 3-4% in stead of the usually described 30-40%. Figure 4 shows the cellulose content of the selected 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, the csr14 transformants showed a cellulose content comparable to WT levels. The cellulose content of the csr10 transformants is clearly correlated to the PCA cluster plot showing transformant csr10-1 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 will not be the basis for the discrimination.

Transformant csr17-8, with a reduction of 50%, shows the lowest amount of cellulose identified in the selected plants. Based on the position of transformant csr17-11 in the PCA plot according to PC3, it was expected that this transformant was most distant from the control plants. However, transformants csr17-3 and 8 have much lower amounts of cellulose. The csr7 transformants show relatively small decreases in cellulose content (25% in comparison to 50% for csr17-3).

FIGURE 4.
Cellulose content in transformed and WT plants. Cellulose content of tuber material was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total CWM (including starch). All measurements were performed in duplicate and standard deviations are represented by the bars.
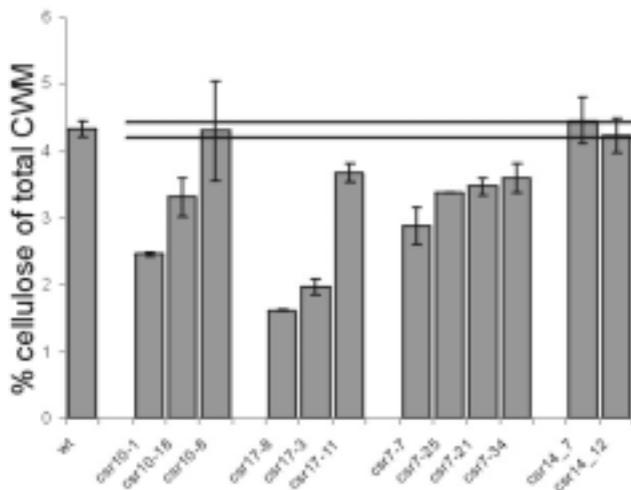
Monosaccharide composition of the various transgenic tubers differs

Table 1 shows the monosaccharide compositions of the different transformants and a WT control. 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).

TABLE 1. Glycosyl residue composition (mol %) of cell wall material isolated from tubers of wild type and *StCesA7, 10* and *17* CSR antisense expressing potato plants. The values represent the average of a duplicate analysis 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
csr10-1	2 ± 0.4	15 ± 1.1	4 ± 0.4	1 ±0.3	21 ± 1.6	57 ± 2.4
csr10-18	3 ± 0.6	12 ± 0.0	6 ± 0.2	2 ± 0.3	36 ± 1.2	41 ± 1.1
csr10-8	3 ±0.5	12 ± 0.1	7 ± 0.2	2 ± 0.2	31 ± 0.8	44 ± 0.9
csr17-8	3 ± 0.3	11 ± 0.5	5 ± 0.4	0	33 ± 1.0	47 ± 0.1
csr17-3	4 ± 0.7	11 ± 0.1	6 ± 0.9	0	35 ± 3.5	44 ± 3.4
csr17-11	3 ± 0.3	13 ± 0.4	7 ± 0.4	2 ± 0.3	39 ± 0.4	36 ± 1.2
csr7-7	3 ± 0.7	11 ± 0.5	6 ± 0.2	2 ± 0.1	32 ± 1.3	46 ± 1.4
csr7-25	5 ± 1.1	11 ± 0.2	7 ± 1.3	2 ± 0.2	31 ± 1.8	44 ± 4.2
csr7-21	3 ± 0.8	12 ± 0.2	7 ± 0.4	2 ± 0.1	39 ± 2.5	37 ± 3.8
csr7-34	3 ± 0.2	12 ± 0.8	6 ± 1.0	6 ± 5.2	33 ± 0.9	41 ± 4.2
WT	2 ± 0.3	11 ± 0.2	6 ± 0.1	2 ± 0.2	38 ± 1.7	42 ± 2.7

For the csr10 transformants line number 1 (containing the lowest amount of cellulose for this construct) shows an obvious increase in uronic acid in addition to decreased levels of galactose and mannose. The other two transformants which show cellulose levels closer to WT do not show significant changes for the respective monosaccharides. Transformant csr17-8 shows modifications which are comparable to csr-10. Additionally the other two csr17 transformants show a variation of decreasing uronic acid levels to be correlated with increased cellulose levels.

The csr7 transgenic tubers show varying small alterations in their cell wall monosaccharides. Transformants #7 and 25 show a decrease in galactose that is combined with small increases in uronic acid. Transformant #21 shows a decrease in uronic acid and no change in galactose. Transformant #34, which does not show a significant decrease in cellulose, shows a decrease in galactose.

Comparison of the cellulose levels in the various transformants with the monosaccharide composition shows that in the csr10 and 17 transformants the lines with the lowest amount of cellulose gives the most obvious changes in pectin composition. The csr7 transformants which have much lower decreased levels of cellulose show mostly small changes in their pectin composition.

DISCUSSION

In this paper, we have down-regulated the RNA expression levels of specific potato *CesA* genes by expressing antisense constructs containing the CSR regions of four potato *CesA* cDNAs. Transformants, generated by *Agrobacterium tumefaciens*-mediated transformation of potato containing the csr7, 10 and 17, showed clear reductions in cellulose content. For most transformants these reductions were confirmed by decreased expression levels of the corresponding *CesA* (compare Figure 3 and 4), demonstrating that the use of only the CSR in an antisense construct is sufficient to modulate cellulose levels. However, csr14 transformants showed no changes in cell wall composition. Previously, we showed that potato plants in which the full-length *StCesA14* cDNA clone was used for sense and antisense expression showed a modified cell wall composition (Chapter 3). One explanation for this apparent discrepancy is that the modifications observed in the full-length sense and antisense transformants are a result of a down-regulated expression of multiple potato *CesA* genes rather than solely the endogenous *StCesA14*. The csr14 construct may provide more specific down-regulation of *StCesA14*. However, for two of the csr14 transformants RT-PCR analysis showed that in these plants *StCesA14* expression levels were similar to WT. This suggests that the antisense csr14 construct is unsuccessful in reducing *StCesA14* which would directly explain the fact that none of the csr14 transformants could be distinguished from WT plants by FTIR and PCA.

Apart from the reduction in cellulose content, the csr7, 10 and 17 transformants also show changes in monosaccharide composition. Both csr10 and csr17 transformants show that the decrease in cellulose is correlated to increases in the uronic acid content and a decrease in galactose. These modifications are consistent with results from several other plant systems in which cellulose is deficient (Burton *et al*., 2000, Fagard *et al*., 2000, His *et al*., 2001, Shedletzky *et al*., 1990 and 1992). More or longer homogalacturonan chains have been suggested to form a stronger Ca^{2+}-bridged pectate network, to compensate for the reduction in cellulose, the major load-bearing component of the cell wall. Further, a reduction of galactan may result in a less branched pectin, facilitating the formation of Ca^{2+} cross-links. However, other potato transformants with similar decreased cellulose levels had no changes in the pectin composition (Chapter 3). This may indicate a delicate balance between cellulose and pectin biosynthesis. The transformants thus seem to be able to resist a 50% reduction of cellulose in potato tubers without altering pectin composition. This is in contrast with the *Arabidopsis* mutants (Arioli *et al*., 1998, Desprez *et al*., 2002, Fagard *et al*., 2000, Scheible *et al*., 2001) and the transformed tobacco and *Arabidopsis* plants (Burn *et al*., 2002; Burton *et al*., 2000).

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. As described by Vergara and Carpita (2001), these three clusters are likely to contain all

primary cell wall cellulose synthases. Mutant and antisense plants with loss of 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 cell wall cellulose. Some of the genes which are not present in these clusters have been assigned a role in secondary cell wall cellulose biosynthesis (Burn *et al.*, 2002; Taylor *et al.*, 1999; Taylor *et al.*, 2000; Wang and Loopstra 1998; Wu *et al.*, 2000). Further, Taylor *et al.* (2000) have shown the existence of a physical interaction between the *irx1* and *irx3* gene products, suggesting that they may be present in one cellulose synthase complex. This has also been suggested for the synthesis of primary cell wall cellulose which may require *Arabidopsis CesA* genes from the different clusters (Desprez *et al.*, 2002). The close homology of the potato *CesA* sequences to the corresponding genes from *Arabidopsis thaliana* and the fact that the potato genes were isolated from a single cDNA-library generated from swelling stolon tips supports the hypothesis that as in *Arabidopsis* these potato *CesA*s may belong to the same cellulose synthase complex which is active during tuber development. Since potato tubers contain predominantly parenchyma tissue this complex is probably involved in primary cell wall biosynthesis.

The cellulose synthase complex is believed to be present as a rosette terminal complex (TC) which was first described in plants by Mueller and Brown (1980). The composition of these TCs is still unclear and evidence is accumulating that subunit TCs are heterogeneous assemblies, consisting at least of multiple *CesA* proteins, sitosterol-β-glucoside transferase, and an endo-glucanase (Peng *et al.*, 2002; Read and Bacic, 2002). In fact, it is possible that TC subunit composition varies in time i.e. a TC may acquire new subunits at different developmental stages. The possible importance of the oxidative dimerization between two *CesA* subunits for rosette assembly (Kurek *et al.*, 2002) further supports this hypothesis. It is still unclear how many *CesA* proteins can participate in one cellulose synthase complex, and whether all *CesA* subunits are equally important. Even though the lack of modifications in the csr14 transformants seems to be an effect of limited down regulation (confirmed in only two transformants) this can also be the result of the *StCesA14* playing a minor role in the corresponding cellulose synthase complex, or the presence of a redundant *CesA* protein in the complex which is still normally expressed. A similar result was found in antisense *CesA2 Arabidopsis* plants which showed only a very mild phenotype and no significant decrease in their cellulose content (Burn *et al.*, 2002).

Concerning the similarities in the modifications found for the csr10 and csr17 it is not unlikely that both these proteins are involved in the formation of the same cellulose synthase complex. Additional PC analysis attempting to separate the csr10 and csr17 transformants was not successful indicating that both series of transformants have the same alterations in their cell wall polysaccharides as was confirmed by the sugar composition analysis. Similar comparisons between other sets of transformants (e.g. csr10 and csr7) showed different kinds of modifications. This in addition to the low reduction in cellulose levels suggests that the down regulation of *StCesA7* may affect a

different (kind of) TC complex or that the *StCesA7* has a different function as *StCesA10* and *StCesA17* in the same complex.

The transgenic lines reported here have demonstrated the possibility to down-regulate specifically the different potato *CesA* genes in potato tubers. These lines will be useful materials to reveal the specific function of their 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. The study from Burn *et al*. (2002) additionally showed the difficulty to extrapolate the results from antisense plants and mutagenised 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. An advantage of the antisense approach is that it ensures that one is studying the effect of a decreased expression of the target gene.

EXPERIMENTAL PROCEDURES

Vector construction and transformation of potato plants
Four antisense constructs were made based on the CSR region of the four potato *CesA* clones and the pBIN20gbss vector, which is derived from the pBIN20 (Hennegan and Danna, 1998) as described in Chapter 3. The CSR region of the four cDNA clones was PCR amplified with specific primers; *StCesA*10; Forward caggcaggctctatacgggt, Reverse ccattcagtcttgtcctcgta, *StCesA*17; Forward gaggcaagcgctttatggatat, Reverse ccccattcagttttgtcttcata, *StCesA*7; Forward gaggcaagcccctttatggttat, Reverse ccccattctgtttttgtcttcata, *StCesA*14; Forward cagctttatatggttatgaacct, Reverse ccccattctgatttatcttcata. 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. The constructs were checked for sense or antisense orientation of the CSR 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.

FTIR screening
Vibratome sections (70 µm) were taken from freshly harvested wild-type and transgenic tubers and further processed and analysed as described in Chapter 3.

RT-PCR method
RT-PCR was performed as previously described in Chapter 3 using the following primer/Tm/cycle-number combinations. *StUGE51* primers, F(5'-ttggtaaccccaaggatttt-3'), R(5'-tttagcggacggtcatgttgttgc-3') Tm=55°C, 28 cycles. *StCesA14* primers, F(5'-gatggaaatgagttgccacgacta-3'), R(5'-ccacttcaaccgtccattgtatcc-3') Tm=58.5°C, 22 cycles. *StCesA7* primers, F(5'-ggccgaaatggtgttgctgttgt-3'), R(5'-cctatcgggcatgcagtagacg-3') Tm=67°C, 23 cycles. *StCesA10* primers, F(5'-gtgttgtttaacaggcaggctctatac-3'), R(5'-aggcttagtggatgcgggaatg-3') Tm=58.3°C, 23 cycles. *StCesA17* primers, F(5'-ttctttttatcggtggtcgttttcc-3'), R(5'-tgcgggtgtcagcggtcatc-3') Tm=51°C, 31 cycles.

Isolation of cell wall material from potato tubers
For the isolation of CWM, one tuber from each of two to three transformed plants were pooled and ground to a fine powder in liquid nitrogen. 10 g of this tuber material was used for cell wall isolation as described in Chapter 3.

Analysis of the cellulose content
50 mg 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 for determination of the cellulose content colorimetrically using anthrone in H_2SO_4 as a coloring agent according to Updegraff (1969). These analyses were performed in duplicate.

Analysis of the neutral sugars
For the de-starched CWM (Chapter 3) the neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples (for CWM and the residue) 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. These analyses were performed in duplicate.

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5

Altered expression of two UDP-Glc-4-epimerases in potato (Solanum tuberosum L.) shows a tissue specific modification of the cell wall composition

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SUMMARY

Two potato UPD-Glc-4-epimerase (UGE) genes (*StUGE45* and *StUGE51*) were isolated from a potato cDNA library using the *A. thaliana UGE1* cDNA as a probe. Both cDNA clones show high sequence similarity with other plant *UGEs*. Expression analysis showed that while both genes are expressed in most plant tissues the *StUGE51* is higher expressed in older tubers, flowers, stems and *in vitro* plants. Transgenic plants were generated to examine the effect of modified UGE expression levels on cell wall bound galactan levels in potato. Sense expression of the two *StUGE* clones in potato gave plants with no overt developmental phenotype. In contrast to WT plants, a large number of the transformants had the ability to overcome the toxic effect of galactose in the culture medium during *in vitro* growth. The UGE45 transformants showed a clear correlation of increased UGE RNA expression levels with this galactose tolerance. These findings are consistent with those in *A. thaliana* plants with increased *UGE1* levels. Additionally, the elevated *UGE* expression in the transformants resulted in an altered monosaccharide composition. The potato tubers showed increased levels of cell wall bound galactan and a decrease in uronic acid content. This indicates that the amount of galactan in the cell wall can be altered by modulating the UDP-Gal pool size. This effect was more profound for the UGE45 transformants than for the UGE51 plants. The fact that elevated expression levels of the two genes can have different effects suggests that the two *StUGE*s have a different function in plant development.

INTRODUCTION

During the process of cell wall biosynthesis the different glycosyl transferases use varying nucleoside-diphosphate (NDP) sugars to assemble the different cell wall polysaccharides (Feingold and Avigad, 1980; Feingold and Barber, 1990; Mohnen, 1999; Gibeaut, 2000). The availability of the NDP-sugars is thus an important factor in determining the final polysaccharide composition. Nevertheless, it is unclear to what extent modifications in the pool size of a particular NDP-sugar can affect the presence or structure of cell wall polysaccharides.

The UDP-glucose-4-epimerase (UGE) catalyses the reversible conversion of UDP-galactose (UDP-Gal) to UDP-glucose (UDP-Glc) (Maitra and Ankel, 1971). The gene encoding UGE was initially isolated from bacteria, yeast and humans (Citron and Donelson, 1984; Daude *et al.*, 1995; Lemaire and Müller-Hill, 1986). Mutagenized organisms harbouring a deficient *UGE* show different effects as a result of this disorder. For instance, yeast and *E. coli* mutants with a defective *UGE* are unable to grow on galactose-containing media (Douglas and Hawthorne, 1964; Yarmolinsky *et al.*, 1959). Complementation studies with these mutants were used as a tool to clone plant *UGE*s from *Arabidopsis*, pea and guar (Dörmann and Benning, 1996; Joersbo *et al.*, 1999; Lake *et al.*, 1998). For these, proof of function was delivered. Later,

additional sequences were obtained from various Arabidopsis sequencing projects suggesting that different forms of UGE exist (*UGE1, 2, 3, 4* and *5*, Reiter and Vanzin, 2001).

In their review on NDP-sugar conversion pathways in plants, Reiter and Vanzin (2001) showed the complexity of the biosynthesis of the various UDP-sugars related to the synthesis of plant cell wall polysaccharides. Most of the UDP-sugar pools are directly or indirectly linked to each other, either by one-way or reversible reactions. One UDP-sugar can thus be a product from various reactions. This implies that, for most UDP-sugars, it is difficult to alter the pool size by modifying the expression of a single gene. The UDP-Gal is synthesised by only two routes; (i) from UDP-Glc by UGE, and (ii) from externally added D-galactose by galactokinase and galactose-1-phosphate uridyl transferase (Reiter and Vanzin, 2001). Thus, if galactose is not externally supplied to growing plants, UGE is the factor determining the UDP-Gal pool size. Therefore, it is likely that modulation of UGE RNA expression levels is an efficient approach to modify the level of UDP-Gal, and consequently to obtain plants with altered levels of cell wall bound galactan.

This approach of modifying levels of cell wall bound galactan by altering UDP-Gal levels contrasts with the approach used by Sörensen *et al*. (2001) to decrease cell wall bound galactan. They showed that by introducing a fungal endo-1,4-β-D-galactanase, rhamnogalacturonan I with a reduced amount of galactan hairs could be engineered. This reduction did not seem to have an impact on plant growth or morphology, suggesting a minor role of the galactan side chains during plant development. The use of UGE for genetic modification enables the possibility to obtain decreased as well as increased levels of galactan. However, reduction or increase of the UDP-Gal pool size is likely to affect also other galactosyl-containing polymers such as xyloglucan or galactomannan. Because the various UDP-sugar pools are connected, also the fine structure and amount of other, galactosyl-lacking, polysaccharides may be affected.

The *Arabidopsis UGE1* gene has been used for sense and antisense expression, resulting in a reduction of UGE activity to 10% of the WT level in antisense plants suggesting that the *UGE1* is the predominant epimerase in *A. thaliana* (Dörmann and Benning, 1998). In plants cultured under normal growth conditions this reduction did not have any impact on the UDP-Glc and UDP-Gal levels nor on the amount of galactose incorporated in cell wall polysaccharides. However, plants with a modified UGE activity showed alterations upon growth on galactose-containing media. Increased incorporation of galactose in cell wall polysaccharides was found in WT plants and plants with decreased UGE activity. This was not found in plants with an increased UGE activity. In addition, plants with increased UGE activity also managed to overcome the toxic effect of the added galactose (Loughman *et al*., 1989; Maretzki and Thom, 1978; Yamamoto *et al*., 1988) and developed normally.

The fact that plants with a decreased UGE activity showed no cell wall changes at normal growth conditions might indicate that a further decrease (below 10%) is necessary to modify the galactan content of the cell wall. Such a reduction may require

the down regulation of more than one *UGE* (*UGE2, 3, 4* and *5*, Reiter and Vanzin, 2001). So far, it is unclear if the various *UGEs* are differently expressed in different plant tissues and developmental stages. The availability of these results and the comparison with multiple plant systems could aid in assigning a particular function to the different *UGE*s or reveal them to have the same function in particular tissues or during specific developmental stages. This is also indicated by the *A. thaliana* mutant with a defect in the *UGE4* gene. This mutant is distorted in root hair development, which is most likely a result of the mutation interfering with synthesis of galactosylated glycans such as arabinogalactan-proteins (AGPs) (Reiter and Vanzin, 2001).

Apart from possible variations in presence and function of *UGE*s, it is unclear if the observed effects of modulating UGE activity are specific for *Arabidopsis thaliana* or tissue-specific. The fact that e.g. potato tuber cell walls contain more cell wall bound galactan in comparison to the *Arabidopsis* plant indicates that UDP-Gal pool size can differ from species to species. To study the effect of altering the UDP-Gal pool size in the galactan-rich potato tuber cell walls we have modulated the RNA expression levels of two putative potato *UGE*s.

RESULTS

Isolation and sequence comparison of two UDP-Glc epimerases from potato tubers

The *UGE1* clone (U22968) from *Arabidopsis thaliana* was used for the screening of a potato cDNA library made from swelling stolon tips. The screening resulted in the isolation of two different cDNA clones, referred to as *StUGE45* and *StUGE51*, which show a clear homology to other plant and bacterial *UGE*s. The dendrogram in figure 1 represents the amino acid sequence homology between the two potato clones and other plant *UGE*s. The dendrogram clearly shows a separation of the *UGE*s in different clusters. The average sequence identity within one cluster of *UGE*s is about 75% while the identity between some of the *UGE*s from the same plant species is only 62%. Thus, these clusters are not plant species specific, but rather indicate differences between the UGE enzymes. Based on the three different clusters one could suggest that, at least in *Arabidopsis*, three different UGE classes are present.

StUGE45 and StUGE51 show different expression patterns in potato plants

Figure 2 shows the Northern blot hybridizations of RNA isolated from young and old leaves, young and old tubers, flowers, stems and roots hybridized with specific probes for *StUGE45* and *StUGE51*. Both genes are highly expressed in flowers, and show moderate to low expression in leaves, stems, roots and tubers. Further, *StUGE51* shows a higher expression (in comparison with *StUGE45*) in old tuber tissue, flowers, stems and roots. In *in vitro* plants, the *StUGE51* is clearly expressed whereas *StUGE45* RNA could not be detected by Northern blotting (see further, figure 4a).
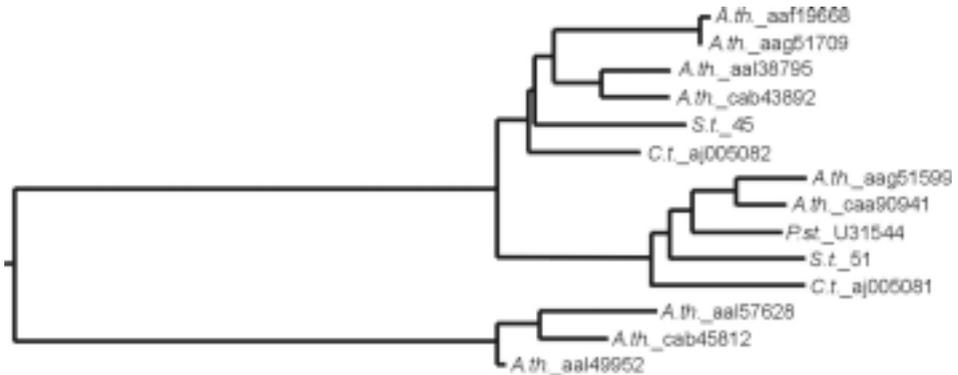
FIGURE 1.

Dendrogram showing the sequence divergence between several plant epimerases.

Plant amino acid sequences suggested or proven to encode UDP-Glc-4-epimerase were used to generate the dendrogram. The following sequences were used: *Arabidopsis thaliana*; aaf19668, aag51709 (*UGE4*), aal38795, cab43892 (*UGE2*), aag51599 (*UGE3*), caa90941(*UGE1*), aal57628, cab45812 and aal49952, *Pisum sativum*; U31544, *Cyamopsis tetragonoloba*; aj005081, aj005082 and *Solanum tuberosum*; S.t._45 (AY197749) and S.t.51 (AY221085). The MegAlign program from the DNA-Star package was used to align the sequences according to the Clustal method using a PAM250 weight table and gap penalties and gap-length penalties of 10.
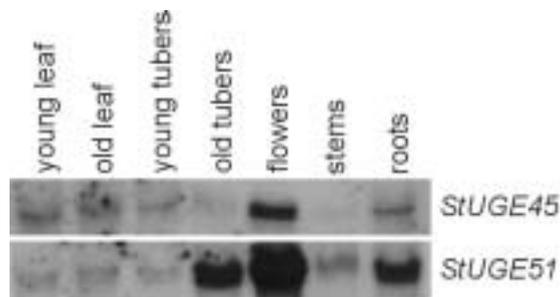
FIGURE 2.

Autoradiograms showing Northern blot hybridisations of RNA isolated from different tissues of greenhouse grown potato plants, hybridized with the UGE specific probes *StUGE45* and *StUGE51*. The lane labelled 'young leaves' contains RNA isolated from 1-3 cm long leaves from the top of young plants. The lane labelled 'old leaves', contains RNA isolated from fully expanded leaves from the lower plant area. The lane labelled 'young tubers', contains RNA isolated from tubers harvested from plants at least 5 weeks before plant senescence initiated. The lane labelled 'old tubers' contains RNA isolated from tubers 2 days after harvest from died plants. The lanes labelled 'flowers', 'stems' and 'roots' contains RNA isolated from these tissues of plants 2 months after planting.

Modulation of UDP-Glc epimerase RNA expression levels in potato plants

Two constructs were made to express the potato *UGE* genes in potato plants in sense orientation controlled by the 35S CaMV promoter (Benfey and Chua, 1990). Besides overexpression, resulting in more UGE activity, these constructs are also likely to generate plants showing co-suppression of the *UGE* gene (and thus decreased *UGE* activity). Transgenic plants showed normal transformation efficiencies (30-40%) compared to control transformations with an empty vector. For each construct 50 transformants were used for further propagation and analysis. The phenotypic and developmental characteristics of the transformants and WT plants were compared during *in vitro* growth on a 2% sucrose medium and during growth of the plants in the greenhouse. None of these transformed plants showed a phenotypic or developmental modification in comparison to wild type plants.

Screening of epimerase transformants for galactose tolerance

To facilitate the selection of transformants with modified UGE activity, a screening method was developed based on the galactose tolerance of the transformed plants. The addition of galactose to growing plants or plant-cell cultures has been shown to inhibit growth (Loughman *et al.*, 1989; Maretzki and Thom, 1978; Yamamoto *et al.*, 1988). Dörmann and Benning (1998) showed that increased UGE activity can induce a tolerance to galactose, whereas the toxicity of galactose becomes more apparent with decreased activity. We have used this phenomenon to screen the potato transformants for modified UGE expression. First, the tolerance to galactose of *in vitro* grown WT potato plants was investigated. The tip of the *in vitro* plants (top 1 cm) was cut and transferred to MS medium in which sucrose was replaced by varying concentrations of galactose. This clearly showed that the presence of galactose inhibited root formation of the plants, and that the plant deteriorated within a period of 2 weeks. Subsequently, fresh tips were transferred to MS medium containing 2% sucrose. After rooting and continued growth of the plant (two weeks), a liquid galactose MS medium was added. Two days after the addition of galactose the plants started to show a browning of the root tips (Figure 3). This browning proceeded and finally appeared in all roots, while growth of the plants was stunted. Different concentrations of galactose were tested which showed that the severity of the effect increased from only browning of the root tip with normal growth (low concentration of galactose), to fast deterioration of the plant with increasing galactose concentrations. The use of a final concentration of 2% galactose seemed to give the best result for our purpose since all WT plants developed a browning root tip within a few days, but they were still able to grow.

For the screening of the transformants, all plants were grown at this 2% galactose concentration in order to distinguish plants with increased and reduced UGE expression. About 19% of all transformants (UGE45 and UGE51) showed the same process of browning and reduced growth as the WT plants (Figure 3, WT and UGE45-9). Nevertheless, 8% of the plants showed a faster and more severe response to galactose. Additionally, 3% of the plants showed the development of many root hairs

close to the root tip (Figure 3, UGE45-54). Apart from the plants showing an equal or more severe response, 48% of the plants also showed no response to the galactose and seemed to develop normally (Figure 3, UGE45-43). Apparently, the toxic effect of galactose is neutralized in these plants.
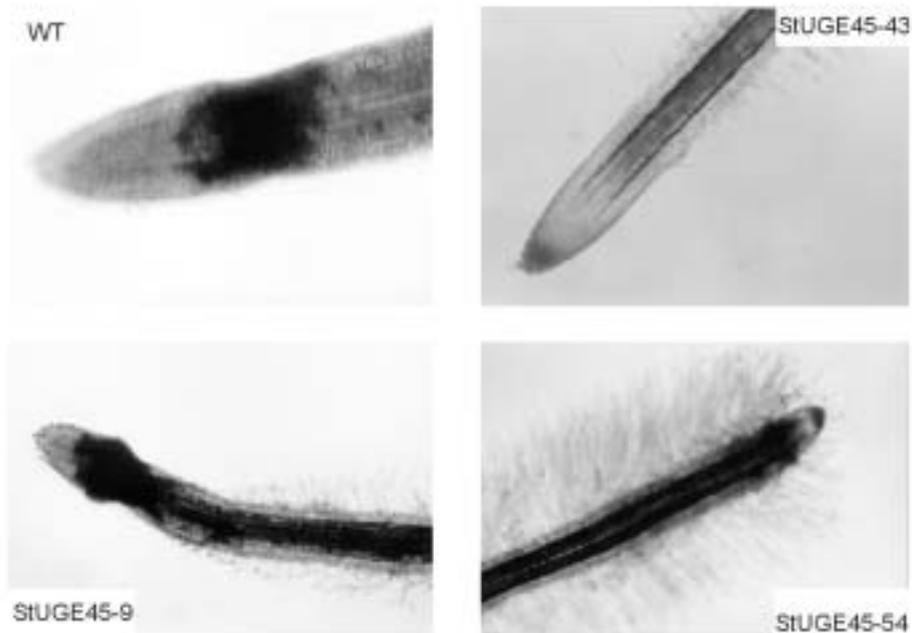

FIGURE 3.
Response to galactose of WT plants and plants with a modified UDP-Glc epimerase expression. WT and transformed plants were grown on normal MS medium containing 2% sucrose (w/v) for two weeks. Subsequently, a soluble MS medium containing 9.3% galactose was added, resulting in a final galactose concentration of 2%. Modifications in root morphology were studied for 7 days after which the plants (only stems and leaves, not the roots) were harvested for RNA isolation. In comparison with the response found in WT plants (browning of the root tip) several transformants showed no response (StUGE45-43), a similar response (StUGE45-9), or a more severe response including severe root hair growth (StUGE45-54).

To study if the different response of the transformants to galactose was indeed a result of modified UGE RNA expression, RNA was isolated from these plants and used for Northern blot analysis (Figure 4a). In WT plants only StUGE51 expression was detected. The transformants showed varying expression levels of the corresponding UGE. Since in WT StUGE45 was not detected only transformants with increased (e.g. number 43 and 59) and undetectable (as in WT) expression (e.g. number 60) could be assigned. The UGE51 transformants showed also increased levels (e.g. number 16) and decreased levels (e.g. number 29) of expression in comparison with the WT plants.

FIGURE 4.
a, Autoradiograms showing RNA blots from RNA isolated from the complete aerial parts of *in vitro* grown plants to which the galactose medium was added. Different UGE45, UGE51 and WT plant RNA samples were hybridised with the respective UGE-specific probes.
b, Overview of correlation between response to galactose and RNA expression levels in various transgenic *in vitro* plants. In the bars representing the tolerant and non-tolerant UGE45 and UGE51 transformants, the number of plants with increased (grey), normal (white) and decreased (dashed) UDP-Glc epimerase expression are given.
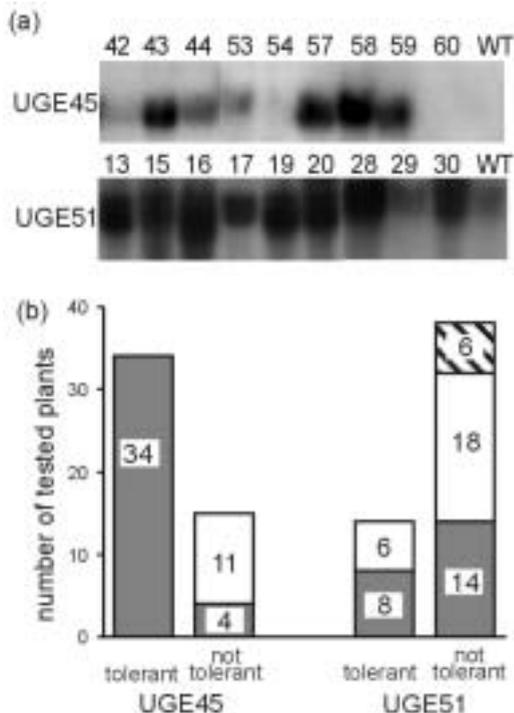
Figure 4b shows the correlation between the tolerance to galactose and the StUGE expression level in the transformants. 69% of the UGE45 transformants are tolerant to galactose and all show (elevated) *StUGE45* expression (which was not detectable in the WT in vitro plants). Those UGE45 plants that are not tolerant to galactose show no UGE expression in most cases (11 out of 15). It thus seems that the tolerance to galactose is correlated to the RNA expression level of the *StUGE45*. The UGE51 transformants did not show this correlation. Only 27% showed a tolerance to galactose of which only 8 out of 14 showed elevated RNA expression levels (the *StUGE51* is actively expressed in the WT *in vitro* plants). Those plants that showed no tolerance to galactose showed elevated (14) as well as normal (18) and decreased (6) levels of *StUGE51* expression. Apparently *StUGE45* and *StUGE51* respond differently to the addition of galactose and only elevated *StUGE45* expression levels can result in detoxification of galactose.

Altered levels of cell wall bound galactan in the UDP-Glc epimerase transformants

Based on the RNA expression levels and tolerance to galactose a number of transformants was selected for the isolation and analysis of CWM. As said, transformants with decreased expression of the *StUGE45* could not be detected and only few UGE51 plants showed decreased expression of the corresponding gene. Therefore we have focussed on transformants showing overexpression of the UGE. CWM was isolated from *in vitro* plants grown both on normal as well as on galactose-containing medium. Table 1 shows the sugar composition of CWM isolated from *in vitro* plants grown on normal medium. Apart from the monosaccharide composition the RNA expression level is also indicated. Comparison of WT and transformed plants shows a decreased galactose content in the transformants with an increased *UGE* expression, especially for the UGE51 plants. Further, this decrease in galactose appears to be correlated to a small increase in uronic acid. The modulation of *UGE* expression seemed to have no obvious effect on the other monosaccharides.

TABLE 1. Monosaccharide composition (mol %) of cell wall material isolated from *in vitro* plants grown on normal culture medium. To facilitate the comparison of the different sugars the Glc has been set zero. The symbols in the RNA column represent small increases (±) up to large increases (+++) in RNA expression level of the corresponding UGE in comparison to the WT (determined on RNA isolated from the complete aerial plant part).

	Rha	Ara	Xyl	Man	Gal	UA	RNA
UGE45-1[*]	3	8	17	4	22	45	+++
UGE45-43	2 ± 0.5	8 ± 0.8	18 ± 2.3	4 ± 0.0	22 ± 7.5	46 ± 3.9	++
UGE45-9[*]	3	9	11	2	27	48	+
UGE51-15	2 ± 0.1	9 ± 0.2	17 ± 1.3	4 ± 0.2	22 ± 3.3	46 ± 1.6	±
UGE51-16	2 ± 0.3	9 ± 1.5	18 ± 1.3	4 ± 0.1	18 ± 3.0	49 ± 2.6	+
UGE51-19	2 ± 0.1	12 ± 0.3	18 ± 2.3	4 ± 0.2	19 ± 2.7	46 ± 0.2	+
WT	3 ± 0.3	8 ± 0.8	15 ± 1.5	4 ± 0.4	27 ± 2.7	42 ± 5.7	

[*] The values for these transformants concern single measurements and thus not include standard deviations.

CWM was also isolated from plants grown on galactose (2%) containing medium. Sugar composition analysis (Table 2) clearly shows that both UGE45 and UGE51 transformants have decreased levels of galactose in combination with increased uronic acid levels. Comparison of the plant specific results from Table 2 (grown on galactose) with those from Table 1 (grown at normal medium) shows that in the WT plants addition of galactose has resulted in small increases of galactan and decreased uronic acid levels. Another difference, also found in the UGE51 transformants, is the increase in cell wall arabinose upon galactose addition.

TABLE 2. Monosaccharide composition (mol %) of cell wall material isolated from *in vitro* plants treated with a galactose solution. To facilitate the comparison of the different sugars the Glc has been set zero. The symbols in the RNA column represent small increases (±) up to large increases (+++) in RNA expression level of the corresponding UGE in comparison to the WT (determined on RNA isolated from the complete aerial plant part).

	Rha	Ara	Xyl	Man	Gal	UA	RNA
UGE45-1	2 ± 0.2	10 ± 0.8	18 ± 2.0	3 ± 0.6	19 ± 3.8	48 ± 3.0	+++
UGE45-43	2 ± 0.5	10 ±0.1	20 ± 2.3	4 ± 0.3	20 ± 1.4	44 ± 0.9	++
UGE45-9	2 ± 0.3	16 ± 0.2	12 ± 0.4	3 ± 0.1	19 ± 2.0	48 ± 1.9	+
UGE51-15	2 ± 1.0	15 ± 0.7	17 ± 5.5	3 ± 0.0	20 ± 3.7	43 ± 0.1	±
UGE51-16	2 ± 0.0	15 ± 0.3	18 ± 5.4	4 ± 0.5	22 ± 6.8	39 ± 1.3	+
UGE51-19	3 ± 0.0	21 ± 0.9	17 ± 1.9	3 ± 0.0	19 ± 0.4	38 ± 0.6	+
WT	2 ± 0.2	13 ± 0.5	17 ± 1.4	3 ± 0.4	30 ± 2.8	36 ± 0.7	

Potato tubers show a different effect compared to the in vitro plants

As reviewed by Mohnen (1999) pectin composition can vary during developmental stages of the plant and between different plant tissues. From previous experiments we know that potato tuber material is rich in galactan, compared to other plant parts. This suggests that the UDP-Gal pool size in tubers is higher. It is likely that this is related to UDP-Glc epimerase expression. Indeed, *StUGE51* shows a higher expression in potato tubers in comparison to leaf and stem tissue or *in vitro* plants (Figure 2 and 4). The difference in expression between young and old tuber tissues may also be linked to a more abundant presence of galactose in later developmental stages of the potato tuber (Bush et al., 2001).

TABLE 3. Monosaccharide composition (mol %) of cell wall material isolated from potato tubers. To facilitate the comparison of the different sugars the Glc has been set zero. The symbols in the RNA column represent small increases (±) up to large increases (+++) in RNA expression level of the corresponding UGE in comparison to the WT, determined on RNA isolated from tubers.

	Rha	Ara	Xyl	Man	Gal	UA	RNA
UGE45-1	2 ± 0.2	10 ± 0.6	4 ± 0.6	2 ± 0.4	51 ± 1.7	30 ± 0.0	+++
UGE45-43	2 ± 0.1	10 ±0.1	4 ± 0.0	2 ± 0.1	49 ± 0.8	33 ± 0.7	++
UGE45-9	2 ± 0.2	12 ± 0.0	5 ± 0.2	1 ± 0.0	46 ± 0.2	34 ± 0.2	±
UGE51-15	3 ± 0.3	11 ± 0.4	4 ± 0.7	2 ± 0.6	45 ± 1.3	34 ± 0.2	+++
UGE51-16	2 ± 0.4	13 ± 0.7	5 ± 0.5	5 ± 3.7	39 ± 0.8	36 ± 1.3	++
UGE51-19	2 ± 0.0	11 ± 0.4	4 ± 0.1	2 ± 0.2	46 ± 0.4	34 ± 0.7	+
WT	3 ± 0.4	13 ± 0.1	5 ± 0.2	2 ± 0.1	41 ± 0.7	35 ± 0.1	

Table 3 shows the sugar composition of CWM isolated from potato tubers of transformed and WT plants. Additionally the *UGE* RNA expression levels in transgenic tubers in relation to the WT tubers is indicated. Comparison of WT values from potato tubers with those of *in vitro* plants shows that tuber CWM has higher levels of galactose and arabinose in combination with lower levels of xylose and uronic acid. This suggests that in potato tubers the rhamnogalacturonan I has more side chains in comparison to the *in vitro* plants.

In comparison with the WT tubers, the tubers from transformants have an increased galactose content correlated with small decreases in uronic acid, which are more profound for the UGE45 transformants than for the UGE51 transformants. This increased galactose content seems to contradict the results found in the *in vitro* plants in which the transformants mostly showed decreased galactose and increased uronic acid levels in comparison to the WT.

DISCUSSION

The screening of a potato cDNA library made from developing tubers with an *Arabidopsis UGE* as probe resulted in the isolation of two potato *UGE*s sharing high sequence similarity with UGE sequences isolated from other plant species. Both *UGE*s were used for sense expression in potato plants, which resulted in plants with mainly increased RNA expression levels. The transformed potato plants show no phenotypic alterations in comparison to the WT plants. This is in line with the *Arabidopsis thaliana* (Dörmann and Benning, 1998) transformants with increased and decreased expression levels of the *Arabidopsis UGE1*, which is most homologous to *StUGE51*.

Northern blot analysis showed that in general the *StUGE51* is higher expressed, in particular in old tubers, flowers, stems, roots and *in vitro* plants. It seems likely that both genes encode an UGE because their elevated RNA expression levels and galactose content of the cell wall are correlated, and because of their sequence homology with *Arabidopsis UGEs* (reviewed in Reiter and Vanzin, 2001).

As in *Arabidopsis*, the potato transformants also show an altered response to externally added galactose. In both plant species an increased UGE expression enables the plant to grow on a galactose-containing medium. In addition, the antisense UGE *Arabidopsis* plants showed a more severe response to galactose. Some of the potato transformants also showed a more severe response to galactose in comparison to the WT plants (UGE45-54 in Figure 3). This might be based on co-suppression of the *StUGE45* resulting in decreased UGE activity levels. However, for the UGE45 transformants this could not be linked directly to decreased expression levels since expression was already very low (undetectable) in the WT plants.

Based on the results in the over-expressing potato plants *StUGE45* seems to play a more predominant role in tolerance to galactose than *StUGE51*. The UGE45 transformants show a correlation between RNA expression and tolerance to the added

galactose which was not found in the UGE51 transformants. Assuming that both genes encode an UGE, an explanation for this difference may find its origin in the very low *StUGE45* expression level in WT *in vitro* plants. The increased expression levels in the UGE45 transformants are relatively much higher than in the UGE51 transformants. This strong increase may be necessary to overcome the toxicity of galactose. Secondly, the obvious correlation between galactose tolerance and the *StUGE45* expression level might be explained when this enzyme has a lower Km value than the *StUGE51*. If the UGE45 has a lower Km value (and thus a higher affinity) for the UDP-Gal substrate it may be more efficient in converting the excess UDP-Gal to UDP-Glc. This subsequently facilitates the transfer of galactose to UDP-Glc and assists in the removal of the toxic galactose. A similar phenomenon has been shown for two different enzymes with UDP-Glc dehydrogenase activity detected in various plant species (Tenhaken and Thulke, 1996). These enzymes, show different Km values for their substrates, but it has to be noted that sequence divergence for these enzymes is larger than for the two UGEs.

The similar response to galactose addition of *Arabidopsis* and potato plants with modified *UGE* expression is the only clear similarity between these transformants. In contrast to the *Arabidopsis* transformants the potato plants showed a modified cell wall composition upon an increased *UGE* expression in *in vitro* plants (grown on normal and galactose-containing medium) as well as in normally grown potato tubers. This clearly indicates that the effects found in one plant species cannot be directly extrapolated to another. The analysis of WT plants shows that, in comparison to the *in vitro* plant walls, tuber cell walls have more galactose and arabinose combined with less uronic acid and xylose. The different wall composition of *in vitro* plants and tubers upon a modified UGE expression clearly indicates that regulation of the UDP-sugar pool sizes occurs in a tissue dependent fashion and is thus likely to play a determining role in the final cell wall composition. This is in line with the results from Keller *et al*. (1999) who inhibited the GDP-mannose pyrophosphorylase in potato plants. While *in vitro* plants grew normally, transfer to the greenhouse changed them dramatically and showed major differences between the transformants and the WT. Further, only leaf cell walls of the transformants showed a reduced mannose content while tuber cell walls, were not altered.

The elevated levels of cell wall bound galactan in the UGE45 and UGE51 tuber tissues fits the hypothesis that the UDP-Gal pool size is a determining factor for galactan deposition in the cell wall. In addition to the increased galactan levels the cell walls show a decrease in uronic acid. This is most likely a secondary effect of the increased UGE activity. As shown in Figure 5 an increased use of UDP-Glc by UGE reduces the substrate for the UDP-Glc dehydrogenase (UGD) to generate UDP-GlcA, which in turn affects the UDP-GalA concentration. A lowered UDP-GalA level may affect the uronic acid content of the wall.

FIGURE 5.
Carbohydrate metabolism of those UDP-sugars involved in plant cell wall polysaccharides.
Abbreviations: AGK1, D-Galactokinase; GAE, UDP-D-glucuronate 4-epimerase; GalT, galactose-1-phosphate uridyl transferase; RHM, rhamnose biosynthetic genes; SUD, soluble UDP-D-glucuronate decarboxylase; UER, UDP-4-keto-6-deoxy-D-glucose 3,5-epimerase-4-reductase; UGD, UDP-D-glucose-4-epimerase; UGE, UDP-D-glucose 4-epimerase; UXE, UDP-D-Xylose 4-epimerase.
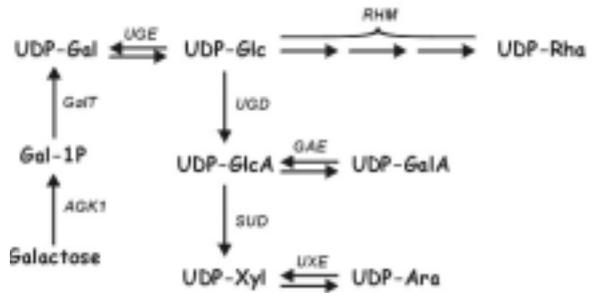
Previously, we have shown that potato transformants with increased levels of cellulose synthesis also indicated the interactive effect of changes in UDP-sugar pool sizes (Chapter 3). In these plants the increased use of UDP-Glc for cellulose production is possibly causing changes in the levels of cell wall bound galactan and uronic acid. However, in this particular case the galactan was reduced and the uronic acid increased, indicating that in a 'normal' situation the UGD has a stronger claim on UDP-Glc than the UGE (e.g. by its higher abundance or activity or stronger affinity for the substrate). These observed changes in cell wall composition are more profound in the tubers of UGE45 than in those of the UGE51 transformants, correlating with the stronger effect of increased *StUGE45* expression on galactose tolerance. Both in *in vitro* plants and tubers the UGE45 transformants show a higher relative increase of the respective RNA level in comparison with that of the UGE51 transformants. This may explain the more prominent increase in cell wall galactose in the UGE45 transformants. As described before, this may also be explained by a higher affinity of the UGE45 for its substrate. The varying effect of increased expression of the two *StUGEs* on galactose tolerance and cell wall bound galactan may indicate an isoform-specific function during plant development.

The effect of decreased galactose content of walls of the transgenic *in vitro* plants is unexpected and much more difficult to explain than the increased galactose levels in the tuber walls. In both cases one would expect the galactose level to increase by the increased UGE activity or at least remain normal if UDP-Gal is already generated in excess in the WT situation. We can only speculate that the different biochemical environment of the UDP-Gal finally determines this specific effect of the increased UGE levels. One explanation may be that increased UDP-Gal levels have triggered the biosynthesis of other galactosyl-containing molecules which are not recovered in our cell wall material. The UDP-galactose is for instance known to serve as a substrate for

the production of mono- and di-galactosyldiacylglycerol, two galactolopids which are important for the complexes of photosystem I and II (Kelly and Dörmann, 2002). These are highly abundant in thylakoids in chloroplasts and thus not present in potato tubers. However, we have so far no additional evidence supporting this hypothesis of additional UDP-Gal uptake.

The modification of UGE transcription levels by sense expression of the two potato UGE sequences has clearly resulted in a modification of the cell wall composition in different plant tissues. The potato tubers of the transgenic plants showed increased levels of galactose. Nevertheless, so far it is still unclear which of the cell wall polysaccharides are affected. Besides in galactan, galactose is present in several other cell wall polysaccharides like rhamnogalacturonan I and II, xyloglucan, galactomannan and arabinogalactan proteins. Further analysis of selected transformants has to reveal which polysaccharides are affected in their fine structure.

EXPERIMENTAL PROCEDURES

Screening of the cDNA library
The UGE clone (U22968) from *Arabidopsis thaliana* was obtained from the Arabidopsis Biological Resource Center (108G20T7, Newman, T.C.). The UGE cDNA was excised using *SalI* and *NotI* and 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). The cDNA fragment was labelled with the rediprime II random prime labelling kit (Amersham pharmacia biotech) and used for the screening of approximately 10^6 phage plaques. Hybridizations were performed for 12 h at 55°C (low stringency because of the screening with a heterologous probe) in modified Church buffer (Church and Gilbert, 1984). The blots were washed twice for 15 min at 55 °C with SSPE solutions of different strength (2x, 1x and 0.5x) containing also 0.1% SDS. Finally three positive phages were identified and the phagemids were excised using the ExAssist Interference-Resistent Helper Phage (Stratagene). This resulted in a pBluescript vector containing the cDNA, which was subsequently sequenced using the T3 (5'-aattaaccctcactaaaggg-3') and T7 (5'-gtaatacgactcactatagggc-3') primers as well as a number of cDNA sequence-specific primers.

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.

Vector construction and transformation of potato plants
Both *StUGE45* and *StUGE51* were used for the construction of a sense construct using the pBIN20 vector (Hennegan and Danna, 1998). The Cauliflower Mosaic virus 35S promoter (Benfey and Chua, 1990) was isolated from the pBI121s (Kuipers *et al.*, 1995) by a *HindIII/BamHI* digestion and ligated into a pBluescript vector. It was subsequently cloned into the pBIN20 vector after a *HindIII/SpeI* digestion of both, generating the pBIN20CaMV. The *StUGE45* and *51* were cloned into the pBIN20CaMV after a *XbaI/XhoI* digestion of both, after which the sense orientation of the UGE cDNAs was confirmed by restriction analysis and PCR amplification.
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 the *in vitro* plants were maintained and propagated *in vitro*. Additionally shoots (in replicates) were transferred to the greenhouse to generate mature plants.

Screening of potato transformants based on tolerance to galactose
To examine the possibility of growing *in vitro* potato plants on galactose containing media tips of WT plants grown (for 2-5 weeks) on MS medium containing 2% sucrose were transferred to MS medium in which the sucrose was replaced by 0 to 12% galactose. On these media the plants were unable to developed a normal root system resulting in deterioration of the plants, which proceeded faster with increasing galactose concentrations. Based on this, tips of plants were transferred to MS medium containing 2% sucrose and after two weeks (during which a normal root system was developed) a liquid medium containing varying concentrations of galactose (1 to 12% of the final volume) was added. The addition of galactose to a final concentration of 2% appeared to be optimal in showing a clear effect (browning of the root tip) of the galactose without further severe deterioration of the plant. For each transgenic line 5 plants were grown and studied for their effect upon galactose addition.

Northern analysis

For RNA isolation both *in vitro* plant material (only the upper-ground part) as well as greenhouse plants (the various WT tissues, and the transgenic tubers) was used. RNA was isolated as described by Kuipers *et al* (1994). 40 µg of RNA was used as described by Sambrook *et al*. (1989) for gel blotting and hybridisation. The membranes were hybridised with ^{32}P-ATP labelled *XbaI-XhoI* fragments of *StUGE45* and *StUGE51*, which selectively hybridised the corresponding gene. A 2.3-kb *EcoRI* fragment of a potato 28 ribosomal RNA gene was used as a control (Landsmann and Uhrig, 1985).

Isolation of cell wall material from *in vitro* plants

Before isolation of CWM, tips of *in vitro* plants were transferred to MS medium containing 2% sucrose and grown for 4 weeks. These plants were used for the isolation of cell wall material and referred to as plants grown on normal medium. A liquid MS medium containing galactose was added to an additional set of plants after one week of growth (resulting in a final galactose concentration of 2%). After another three weeks of growth on this medium the plants were used for isolation of cell wall material and referred to as plants grown on a galactose containing medium.

0.7 Gram of ground (in liquid N_2) 'upperground' *in vitro* plant material was extracted for 3h at RT under continuous shaking in 10 ml of Tris(HCl) solution pH 7.2 at a 50mM final concentration, containing SDS to a final concentration of 1%. The CWM was pelleted by centrifugation at 3000 rpm for 14 min, washed with water, ethanol and aceton and finally air-dried.

Isolation of cell wall material from potato tubers

For each isolation of CWM 10 g of ground (in liquid N_2) tuber material was extracted for 3h at RT under continuous shaking in 25 ml of Tris(HCl) solution pH 7.2 at a 50mM final concentration, containing SDS to a final concentration of 1%. The CWM material was pelleted by centrifugation at 16000 rpm for 14 min; subsequently the residue was washed with water, ethanol, aceton and air-dried. 300 mg of isolated cell wall material 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 starch degradation the CWM was precipitated in 70% ethanol and washed with acetone.

Analysis of the neutral sugars and uronic acid content

For the CWM the neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984), using inositol as an internal standard. The samples (for CWM and the residue) 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. These analysis were performed in duplo and standard deviations were calculated accordingly.

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Leaf protoplasts and cDNA-AFLP as tools to study primary cell wall biosynthesis in potato

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SUMMARY

An RNA fingerprinting study of potato leaf protoplasts was performed to explore its suitability for identifying candidate genes involved in primary cell wall biosynthesis. Microscopic analysis, using calcufluor white to stain cellulose, showed that the protoplasts generated a new cell wall in the first 18h after transfer to a culture medium. cDNA-AFLP was used to visualize differential gene expression at 5 distinct time-points within these first 18 hours. *In vitro* plants (with and without exposure to severe physical damage) served as controls. Around 8500 transcript derived fragments (TDFs) were visualised which showed varying expression patterns in the protoplasts and controls. In total 156 TDFs were isolated, sequenced and used to search for homologies. Over 50% of these TDFs showed homology to described genes, involved in several general plant processes. However, only one cell wall related TDF (a pectin esterase) was found. Our results indicate that even though the protoplasts actively regenerate a new cell wall, this did not result in highly increased expression of genes involved in cell wall biosynthesis or modification.

INTRODUCTION

The plant cell wall has been studied for many purposes and has shown to be a very complex composite of many polysaccharides and proteins (Carpita and Gibeaut, 1993; Fry, 1995). The various cell wall polysaccharides have been suggested to assemble into two independent networks. The cellulose-hemicellulose network provides strength to the cell wall while the pectin network is probably an important factor in determining the pore size of the cell wall (McCann and Roberts, 1994). The biochemical diversity is exemplified by the hemicellulose and pectin polymers that are represented by many different polysaccharide structures composed of various glycosyl residues which can be linked to each other in numerous ways. The implication of this diversity is that many enzymes are likely to be involved in synthesising and assembling these polysaccharide structures as well as in remodelling the cell wall during expansion or differentiation. However, only a few genes encoding these enzymes, particularly in biosynthesis, have been identified. Most of the genes involved in cell wall biosynthesis that have been cloned to date were identified by screening large mutant collections for aberrant phenotypes (Fagard *et al.*, 2000; Turner *et al*., 2001).

Another approach to isolate and clone genes involved in the process of cell wall biosynthesis is the use of transcriptional analysis. Many of these studies have been performed to date, which have enabled the isolation of transcripts of candidate genes involved in particular biological processes. Only few of these studies were focused on cell wall biosynthesis and modification, such as some on fruit ripening, where gene expression in different stages of ripening was studied (Davies and Robinson 2000) and xylem formation, where expression profiles in different tissue sections were compared

(Hertzberg *et al*, 2001; Whetten *et al*., 2001). In the work on the *Zinnia* mesophyll cell system (Milioni *et al.*, 2001), the process of secondary cell wall biosynthesis can be precisely regulated making this system very suitable for gene expression analysis. The relevance of isolated unknown gene transcripts was validated by the detection of cell wall related genes which were found among these transcripts. The presence and expression pattern of these known genes triggered the further investigation of several unknown sequences based on their expression profile.

Most of the cell wall related studies emphasis the development of secondary walls. Two examples are extensive studies in poplar (Hertzberg *et al*., 2001) and pine (Whetten *et al*., 2001) describing the differential expression of many categories of genes during the process of xylem and wood formation. Additionally Hertzberg and co-workers (2001) showed by hierarchical clustering of the expression patterns that many genes of a specific gene category show the same expression profile. Despite the fact that many genes involved in secondary cell wall biosynthesis will also be active during the synthesis of the primary cell wall, there is clear evidence that inner/outer cell wall-specific genes exist. An example of this is the cellulose synthase *CesA* gene family of which some members have been assigned to either primary or secondary wall biosynthesis (Vergara and Carpita, 2001).

Burgess *et al*. (1983) have shown that protoplasts are particularly suitable for studying cell wall biosynthesis. Even though previous studies suggested that protoplasts are not able to rebuild a normal cell wall (Asamizu and Nishi, 1980; Blaschek *et al*., 1981; Pilet *et al*., 1984; Gould *et al*., 1986), Shea *et al*. (1989) showed that using suitable conditions, and improved enzyme treatment, resulted in cells that were not seriously impaired and retained the capacity to develop a normal cell wall. These results were based on the biochemical and cytological characteristics of the protoplasts and their newly generated cell walls. This study investigates whether protoplasts regenerating a cell wall are a suitable system for identifying genes involved in primary wall biosynthesis using cDNA-AFLP (Bachem *et al*., 1996).

RESULTS

Leaf protoplasts regenerate a new cell wall within 18 hours

After physical and enzymatic treatment of leaves from 4 week old *in vitro* potato plants (*Solanum americanum*), protoplasts were isolated and transferred to a culture medium (t=0). Aliquots were taken at various time-points and studied using light and fluorescence microscopy with and without staining of the cellulose by calcofluor white. Even though the development of a new wall by the protoplasts did not occur in a synchronous fashion, several distinct stages could be identified.

Figure 1 shows representative microscopic images of protoplasts at three different time-points. In the early stages of culturing none of the protoplasts showed staining with calcofluor white (Figure 1a and b), indicating that the cell wall of these cells has been completely degraded. After 18 hours, the first cells staining with Calcofluor White can be recognized. Figure 1c and d show a clear Calcofluor White staining of a cell after 24 hours, the time-point when the majority of the cells seem to have formed a cell wall. These cells will continue growth (figure 1e and f) and after a few days the first cell divisions appear (data not shown).

Identification of expression profiles during cell wall formation

To identify the genes involved in cell wall biosynthesis, cells at various stages of the first 18h (0, 3, 6, 12 and 18h) were used for RNA isolation. We expected all enzymes participating in cell wall biosynthesis to be present during this time period and the genes coding for the enzymes to show an elevated expression, particularly in the early stages. In addition, two controls were prepared, one from normally grown (young) *in vitro* plants and one from the same plants after exposure to severe physical damage (to induce a wound response). The damaged plants are likely to show increased expression of genes related to wound response as for instance stress factors and genes involved in plant defence mechanisms.

The 7 templates (5 time-points and 2 controls) were fingerprinted using 141 primer combinations. On average one primer combination visualizes about 50-70 transcript derived fragments (TDFs) resulting in the visualization of about 8500 TDFs in total. Figure 2 shows an example of representative visualized expression patterns. In Figure 2 TDFs 4_6 (a), 3_2 (b) and 3_1 (c) show an increased expression during the 18 hour period (marked by the arrowhead). This increasing expression during cell wall development is accompanied by high expression levels of the corresponding TDFs in the two controls for TDFs 4_6 and 3_2 and a low for TDF 3_1. The TDFs 1-1 (d) and 4_7 (e) show a decreasing expression in the 18 hour period. The fact that TDF 4_7 shows strongly increased expression in the damaged *in vitro* plant in comparison to the normal plant suggests a correlation to the stress in these plants and thus probably also the protoplasts. It is therefore unlikely to be directly involved in cell wall biosynthesis. Some TDFs showed only expression in the two control templates (TDF 8_8, Figure 2f). These are most likely related to metabolic pathways not activated in single cells.

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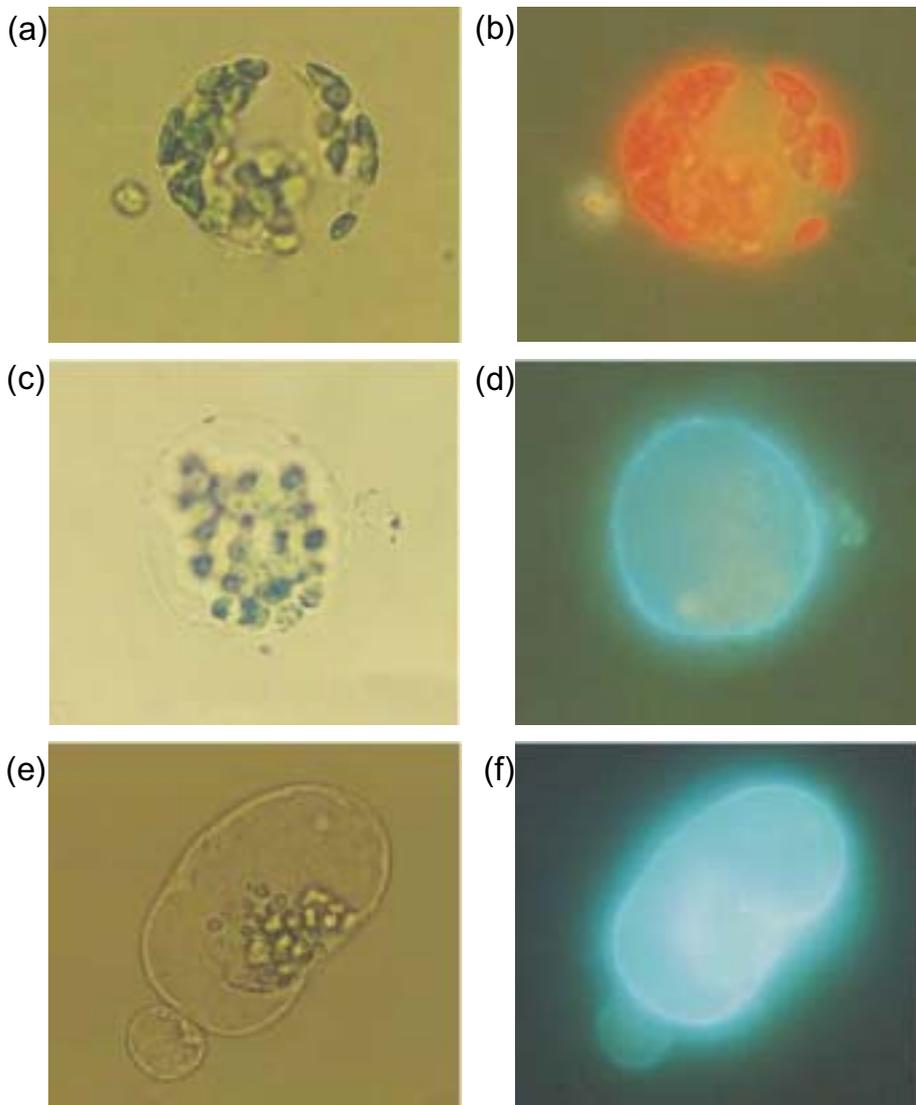
FIGURE 1.
Microscopic images of protoplasts at different stages of development
Protoplasts were stained with Calcofluor white and subsequently studied with normal light (a, c and e) and UV light (b, d and f). Samples were taken at different time-points after transfer of the protoplasts to the culture medium (0h). The pictures show cells at 0h (a and b), 24h (c and d) and 144h (e and f).
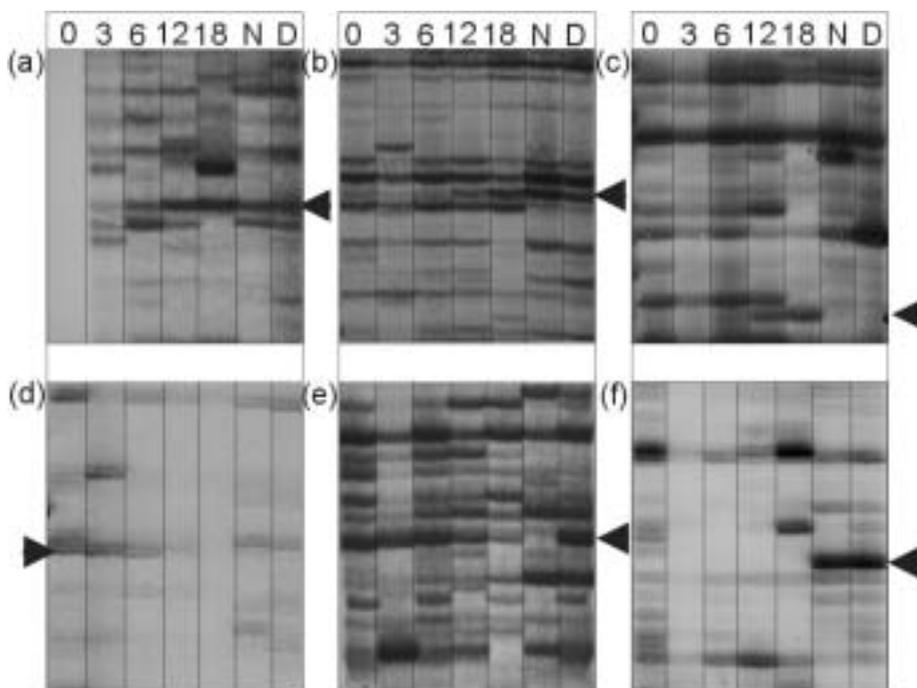
FIGURE 2.
Visualization of transcribed genes for the different protoplast and control templates
cDNA-AFLP images of different primer combinations for the 7 templates. The different lanes each represent a time-point (0, 3, 6, 12 or 18h after transfer to the culture medium) of the protoplast culture or one of the two controls being either the normal (N) or damaged (D) *in vitro* plants (amplification was not successful in lane 0 of panel a. The arrowheads indicate the TDFs (named as gel#_# isolated fragment) described in the text as showing increasing (a: 4_6, b: 3_2, and c: 3_1) or decreasing (d: 1_1 and e: 4_7) expression in the protoplast system. Or no expression in the protoplasts at all (f: 8_8).

Various novel genes appear to be transcribed in the 18h period

156 TDFs were isolated and sequenced based on their expression patterns and on the suitability of the bands for isolation (size, sharpness, etc). We initially focused on the isolation of TDFs showing a modification in their expression during the 18h period. 37% of all isolated TDFs showed an increasing expression, while 27% showed decreasing expression levels in this time period. To enable a more detailed characterization of the whole system, TDFs with continuous expression profiles (18%), transient expression profiles (8%), and TDFs present only in the two control tissues (10%) were also isolated and sequenced.

Sequences obtained were used for homology searches using the BLAST 2 programs (Altschull *et al.*, 1997). For a number of TDFs where a clear sequence homology was identified, the expression profile and possible gene function are shown in Figure 3.

TDF	Size (bp)	Function	TDF	Size (bp)	Function
1_1	296	similar to Drosophila ask2	27_7	420	peptidylprolyl isomerase
1_2	210	lipoxygenase	27_14	245	acetyl-CoA carboxylase carboxyl transferase
3_4	320	IAA amidohydrolase	28_1	490	cinnamyl alcohol dehydrogenase
3_6	600	uridine kinase	28_3	175	auxin response factor 1
3_8	240	anionic peroxidase	28_4	155	NADH dehydrogenase subunit
4_2	600	FH protein interacting protein	28_9	410	cysteine protease
4_3	365	seed maturation-like protein	29_10	390	Cytochrome P450
4_4	115	seed maturation-like protein	29_11	370	ribosomal protein
4_5	270	eukaryotic initiation factor 4	29_12	320	3-isopropylmalate dehydratase
4_7	320	IAA amidohydrolase	29_13	600	beta-cyanoalanine synthase
5_1	250	sucrose-phosphate synthase	29_15	175	wound-induced protein WIN2
5_3	210	propylphenol oxidase	30_2	410	ribosomal protein L41
7_1	420	alcohol dehydrogenase	30_4	350	diacylglycerol kinase
7_2	370	26S proteasome regulatory subunit 8	30_7	600	beta-cyanoalanine synthase
7_3	345	alcohol dehydrogenase	30_10	275	FAR1 – like protein
7_4	185	ribulose biphosphate carboxylase	30_11	260	putative cinnamoyl CoA reductase
7_6	150	stress related protein	31_1	135	auxin induced protein
7_10	205	beta-fructofuranosidase	31_8	225	ERS5 (ethylene responsive) protein
7_11	215	vacuolar metal-ion transport protein MTP1	31_9	215	DEAD BOX RNA helicase RH15
8_1	270	pectinesterase	31_13	155	AIM1
8_3	90	ribulose biphosphate carboxylase	31_16	270	ornithine decarboxylase
25_1	320	small nuclear ribonucleoprotein F	31_17	195	3-hydroxy-3-methylglutaryl coA reductase
25_4	250	cytochrome P450	31_18	115	B12D protein
25_8	190	proline-rich protein	31_19	170	patatin-like protein
25_10	150	peptidylprolyl isomerase	32_1	370	vacuolar ATP synthase
25_13	100	lipid transfer protein	32_3	210	ribulose biphosphate carboxylase
26_1	470	glutaredoxin	32_4	165	tomato mosaic elicitor
26_3	190	lipid transfer protein	32_6	145	GTP-binding protein
26_4	175	ERS (ethylene responsive) protein	32_7	170	tomato mosaic elicitor
26_5	600	putative CCCH-type zinc finger	32_8	155	40S ribosomal protein
26_8	520	casein kinase-like protein	32_11	270	peroxidase
26_11	100	R.X. resistance gene	32_12	195	photosystem I reaction center subunit II p
26_12	530	proton pump interactor	32_14	135	soluble NSF attachment protein
26_13	310	CLIP (cytoplasmic linker protein)	33_1	220	putative berberine bridge enzyme
26_14	300	acyl CoA reductase	33_3	350	AN11
26_16	235	serine acetyltransferase	33_4	235	putative reticuline oxidase
26_17	360	Cytochrome P450	37_1	165	GTX3 probable glutathione S-transferase
27_2	385	starch phosphorylase	37_2	160	glutathione S-transferase
27_4	150	Cytochrome P450	36_1	260	similar to SEC7 protein
27_8	430	putative protein kinase	36_3	260	ripening related protein
			36_4	335	ribulose biphosphate carboxylase

FIGURE 3.
Several TDFs for which sequence homologies were identified
The first column gives the TDFs gel and band number, the size (in bp) is given in the second column, the third column gives the gene function of the gene(s) homologous with this TDF, the last column shows a facsimile of the RNA fingerprint of the TDF.

Only one of the ~150 isolated TDFs could be linked directly to cell wall metabolism. This TDF (also shown in Figure2f), which was only expressed in the two control tissues, showed a high homology to a pectin esterase from *Lycopersicon esculentum*. The putative inferred functions of the other 155 TDFs show no direct correlation with cell wall biosynthesis. All TDFs were assigned to 11 functional categories representing important processes in the plant. Figure 4 shows the distribution of the isolated TDFs over these categories. This data shows that a relatively large number (46%) of the TDFs could not be assigned to a function based on sequence homology (divided in TDFs with no homology to any known sequences (22%) and TDFs with homology to sequences without annotation (24%)). The protein metabolism (including e.g. genes involved in amino acid biosynthesis as well as ribosomal proteins) and catabolism classes each contain 10% of the identified TDFs. Other relatively large groups (respectively 8 and 7%) are the classes defence / stress (e.g. resistance genes and response factors) and growth and development (e.g. a gene related to microtubule

arrangement during cell division). Remarkably the TDFs for which homology to genes related to defence and stress responses were found did not show a specific increased expression in the control tissue which was exposed to severe physical damage. The class of carbohydrate related genes only includes genes which are involved in general polysaccharide biosynthesis (e.g. sucrose synthase) and not cell wall polysaccharide specific genes.

Even though, apart from the pectin esterase, no obvious cell wall-related genes have been identified such genes may be represented in the 46% of TDFs for which no homologies to described genes was found. It is however remarkable that no cellulose synthase or cellulose synthase like genes were identified.
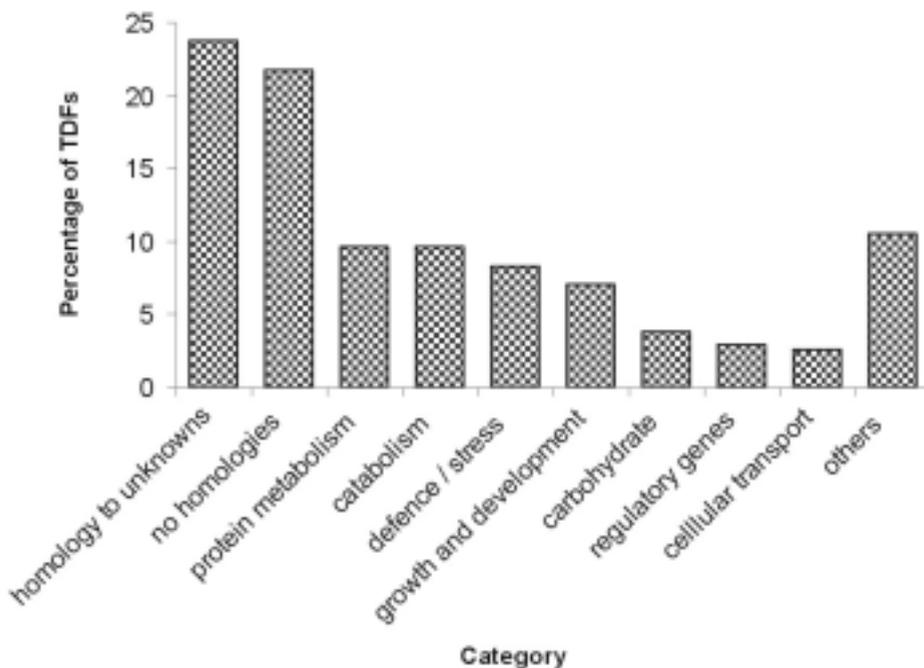

FIGURE 4.
Categories of transcripts identified in the protoplast and control templates
Sequences of the TDFs were studied by BLAST searches and if significant homologies were present genes were assigned to a particular category. The y axis shows the percentage of TDFs being present in a particular category.

Clustering of the transcripts based on transcription pattern and functional category

All 156 cloned and sequenced TDFs were assigned to a gene category and to a particular expression pattern. To study a possible correlation between the transcripts belonging to a category and a particular expression profile all TDFs were sorted based on these parameters (in an MS Excel sheet). This analysis showed that most TDFs were randomly distributed across types of expression and no clear correlation was established between the expression profile and the processes they are involved in. Two trends were however observed. (i) Most of the TDFs involved in carbohydrate metabolism show an increased expression during the 18h period. This was shown for 82% of the TDFs in this category. (ii) On the contrary only few of the TDFs involved in catabolism (13%) showed an increased expression profile.

<div align="center">

DISCUSSION

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To study the process of cell wall formation on a genetic level we have used cDNA-AFLP (Bachem *et al.*, 1996) to fingerprint RNA expression profiles during the process of cell wall formation in potato leaf protoplasts. Visualisation of cellulose in the walls of these cultured cells indicated the formation of a cell wall by the viable protoplasts in the first 18h after transfer to the culture medium. Previous work from Burgess *et al.* (1983) and Shea *et al.* (1989) already showed that protoplasts are able to generate a completely new cell wall marking this system very suitable to study cell wall biosynthesis. Since the formation of a wall by the protoplasts is necessary for further development of the cell and subsequent cell division, it is reasonable to expect that genes involved in cell wall biosynthesis are actively transcribed during regeneration. Ideally, a study on the process of cell wall biosynthesis should be carried out in a synchronous system with a clearly defined time-point at which cell wall biosynthesis is initiated. An example of this was shown by the work from Milioni *et al.* (2002) in which secondary cell wall formation, by isolated *Zinnia elegans* leaf mesophyl cells, was induced precisely 48 hours after addition of growth factors. This highly synchronised system is ideal to select specific time-points for transcript profiling. For studies in poplar and pine different tissue sections, which showed distinctive stages of secondary wall development, were selected (Hertzberg *et al.*, 2001; Whetten *et al.*, 2001). Between these sections differential expression of genes involved in wall synthesis can be expected. These three studies showed to be successful in visualizing the expression of known and putative cell wall related genes. Unfortunately, cell wall biosynthesis by the protoplasts was not very synchronised. The choice for this system in our study was based on the expectation that strongly enhanced cell wall biosynthesis would result in strongly increased expression levels of the genes involved.

Sequence analysis, and subsequent Blast searches, of the isolated TDFs showed that, as in many other RNA fingerprinting experiments, a large number of genes related to

general plant processes were identified such as catabolism, protein metabolism and cellular transport (10, 10 and 3% of the isolated transcripts respectively). The identification of TDFs related to stress and defence mechanisms (8%) is probably a consequence of the enzymatic treatment and physical damage of the cells during the process of protoplast generation and isolation. Despite careful handling, to limit damage of the cells and enable the formation of normal cell walls (Shea *et al*., 1989), the isolation procedure has triggered the expression of genes involved in stress and defence mechanisms.

The expected enrichment in expression of genes related to cell wall biosynthesis was not found in the isolated and sequenced TDFs. The pectin esterase was the only TDF that could be linked to cell wall metabolism because it may have a function during deposition or alteration of the pectic molecules in the cell wall by its ability to modify the pectin esterification. Nevertheless, the pectin esterase is unlikely to be necessary for cell wall biosynthesis in the protoplasts since it did not show transcription in the protoplast culture, but was only transcribed in the two controls. Possibly, the function of this esterase in cell wall related processes is not relevant in isolated cells and it is therefore only transcribed in normal plant tissues.

It is difficult to explain why no cell wall related genes were identified but some hypotheses can be put forward. It may for instance indicate that the expected burst in expression of cell wall related genes has not taken place. It is possible that the apparent increase in cell wall synthesis activities does not require enhanced gene expression levels. One reason for this can be the fact that the protoplasts are isolated from young plant leaves. In these tissues, which are still developing, cell wall related genes may be continuously expressed and not further enhanced during cell wall formation by the protoplasts. Additionally, the proteins involved in cell wall biosynthesis may have a long half life and an increased expression of their encoding genes, at the protoplast stage, is possibly not necessary for regeneration of a new wall.

The identification of many genes related to other cellular processes directly implies that cell wall biosynthesis is not the only process taking place in the protoplast system. Contrary to what was expected this may even not be the most important process taking place in these cells. The TDFs derived from the cell wall related genes may be masked by the more abundant TDFs (both in intensity as well as in differential expression pattern) related to other processes. This may explain why cell wall related genes have not been found.

In stead of biological answers the inability to isolate cell wall related genes may also be imposed by practical drawbacks of the study. Important here is the fact that the cell wall regeneration does not seem to occur in a synchronous fashion and the absence of a clearly defined time-point at which wall biosynthesis is initiated. Further, only few genes involved in cell wall biosynthesis have been identified to date, which decreases the chance of isolating TDFs with sequence homology to these. Nevertheless, the identification of transcripts homologous to for instance a cellulose synthase, cellulose synthase like or xyloglucan *endo*-transglycosylase could have been expected. However,

not all these sequences will be visualised by the used cDNA-AFLP procedure. By using *TaqI* and *AseI* for template preparation and not isolating all generated TDFs an arbitrary selection of transcripts is made which might exclude the transcripts of those genes known to be involved in cell wall biosynthesis. Most likely several of the proposed explanations will be jointly responsible for the fact that no cell wall related genes were identified.

The results from this study obviously show that it will be difficult to identify new genes related to primary cell wall biosynthesis by an RNA fingerprinting approach. Primary cell wall synthesis is basically taking place in all young living tissues and thus difficult to study as an individual process. This is an important difference between primary and secondary wall synthesis. The generation of secondary walls is not an ongoing process and takes only place in particular tissues at particular stages of development. Therefore experiments aimed at the discovery of genes involved in secondary wall synthesis are more likely to be successful. Additionally, also mutant analysis will probably result in the identification of new cell wall related genes. In comparison to gene discovery experiments focussed on primary cell wall development the latter approach seems to have more potential in further clarifying the primary wall biosynthetic machinery.

EXPERIMENTAL PROCEDURES

Protoplast isolation and microscopic analysis

For protoplast isolation 4-week-old *in vitro*-grown shoots from *Solanum americanum* (which has large leaves and showed to have a good yield in protoplast isolation) were used. Isolation of protoplasts was performed as described by Uijtewaal *et al*. (1987) with the small modification that protoplasts were finally transferred to TM2G culture medium (Wolters *et al*., 1991).

Cell wall formation by the protoplasts was studied at several time-points after transfer to the culture medium by bright-field microscopy, and by fluorescence microscopy after staining cellulose in cell walls with Calcofluor White (Sigma, Zwijndrecht, The Netherlands).

RNA isolation and cDNA-AFLP

At 5 different time-points (0, 3, 6, 12 and 18h) after transfer of the protoplasts to a culture medium RNA was isolated from these cells and used for the preparation of the cDNA-AFLP template. Additionally, two control templates were prepared from normally grown (young) *in vitro* plants with and without severe physical damage, resulting in 7 cDNA-AFLP templates. To 25 ml of cell culture (containing 10^6 protoplasts/ml) 5 ml 5X RNA extraction buffer (10% SDS, 50 mM EDTA, 250 mM Tris-Hcl, pH 9) and 20 ml phenol was added. After vortexing the solution was centrifuged (10 min, 3000 rpm, 4°C) and to the top phase 0.6 volumes of isopropanol was added. After 10 min. –80°C and 10 min 3000 rpm, 4°C, the pellet was resuspended in 1 x RNA extraction buffer and stored at –80°C until RNA from all time-points was obtained. This procedure was performed to concentrate the small amount of RNA from the large culture volume of the protoplasts. For the *in vitro* plants, 1-3 gram tissue material was ground in liquid N_2 and immediately extracted with 1X RNA extraction buffer. After phenol-chloroform extraction the RNA was finally (after a first isopropanol precipitation) precipitated with LiCl (2M final concentration) and resuspended in MQ.

cDNA-AFLP template preparation was performed as described by Bachem *et al*. (1998). In total 141 primer combinations of primers with two selective nucleotides were used for the selective amplification and visualisation of the transcripts in the 7 different templates.

Isolation of transcript derived fragments and sequences analysis

Based on the autoradiogram TDFs were excised from the gels, hydrated in TE buffer (pH 8.0) and subsequently transferred to 0.1 X TE buffer for 1 h. 5 µl was used for PCR amplification of the fragment re-using the same primers from the selective amplification. The amplified product was cloned in pGEM-T Easy vectors (Promega Corp, Madison WI, USA) and after checking the cloned fragments on size 3 clones per fragment were sequenced. The DNA-Star software package was used for sequence analysis and sequences were compared with public databases using the Blast 2 programs (Altschul *et al*., 1997)

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7

General discussion

The experiments described in this thesis have been performed to explore the possibilities and effects of altering the cell wall composition in potato. The results of the individual experiments are compared in this chapter with a particular focus on two aims of the thesis work. (i) The examination of different approaches to modify the structure and or amount of the individual polysaccharides, with a particular focus on pectin. This involved interference with cell wall architecture, at different levels of the biosynthetic machinery, as well as the introduction of enzymes able to degrade already deposited pectin. (ii) The use of transgenic plants with a modified cell wall structure to study the biological significance of the individual wall polysaccharides. Additionally these plants will show whether it will be possible to generate plants with tailor-made cell wall structures for industrial applications.

DIFFERENT APPROACHES TO GENERATE PLANTS WITH ALTERED CELL WALL STRUCTURES

The heterologous expression of pectin degrading enzymes

At the start of the work described in this thesis no (putative) pectin synthases were isolated, which favoured the heterologous expression of pectin degrading enzymes to alter pectin structure *in muro*. This appeared to be a very successful approach, used by us and others, and resulted in three transformants (one of which is described in chapter 2 of this thesis) with an alteration in the RG I structure of the tuber cell walls. The introduction of the *e*RGL resulted in a fragmentation of the RG I and showed a decrease in the levels of cell wall bound galactan and arabinan both occurring as side chains of the RG I. The *e*GAL (Sørensen *et al*., 2000) and *e*ARA (Skjøt *et al*., 2002) degraded only the respective RG I side chains and had no effect on the RG I backbone or other attached glycosyl residues. These three transformants with different and specific alterations of the same cell wall polysaccharide have been very useful for hypothesizing the biological significance of the individual RG I structures (see further). Nevertheless, two drawbacks of this approach have to be mentioned.

(i) While the introduction of the *e*GAL, *e*ARA and *e*RGL cDNAs resulted successfully in RNA expression and respective enzyme activities this was not obtained with two similar proteins. The introduction of the cDNAs for a rhamnogalacturonan hydrolase (*e*RGH, Mutter et al., 1996; Schols et al., 1990) and a rhamnogalacturonan acetyl esterase (RGAE, Kauppinen *et al.*, 1995) resulted only in RNA expression of the two genes (Oomen, unpublished results) and did not induce any pectin modifications (studied by Fourier Transform Infra-Red microspectroscopy and antibody labelling). The fact that also Western and dot blot analysis using *e*RGH and *e*RGAE specific antibodies was not successful suggests that a problem occurred during translation of these two enzymes. One should thus take into account that not every single (fungal) pectinase can be expressed successfully in the plant.

(ii) Transformants in which *e*ARA was expressed under control of the granule-bound starch synthase promoter (Visser *et al*., 1991) and directed towards the apoplast showed a severe phenotype (Skjøt *et al*., 2002). These plants produced no side shoots, flowers, stolons and tubers. Interestingly, a pre-deposition strategy where the arabinanase was targeted to the site of pectin biosynthesis, the Golgi apparatus, resulted in transformants (*eG*ARA) with a phenotype indistinguishable from WT and development of normal tubers with a 69 % reduction of RG I-associated arabinosyl groups (Skjøt *et al*., 2002). This suggests that the severe phenotype of the apoplastic *e*ARA transformants is not an effect of the hairy regions lacking arabinan side chains, but related to secondary effects of arabinanase activity at the apoplast. The conditions for expressing and targeting the pectinases of interest may thus be determining factors in obtaining the desirable modification in the cell wall.

It will be difficult to circumvent the problems with translation, transcription or processing of introduced genes and their derivatives. Nevertheless, the study of the Golgi-targeted arabinanase (Skjøt *et al*., 2002) showed that some of the other problems caused by the introduction of a heterologous enzyme can be minimised by careful design of the experiment regarding regulated expression (choice of promoter) and protein targeting. These experiments in addition to the availability of a large scale of pectin (and other wall polysaccharide) modifying enzymes indicate the applicability of this approach to generate specific alterations in cell wall polysaccharide structures in future experiments.

The manipulation of the biosynthetic machinery
In comparison with the post-depositional degradation of pectic polymers by heterologous enzymes an interference with biosynthesis of these polysaccharides (thus at a much earlier stage) seems to be more efficient. Additionally, the degrading enzymes can not be used for studies in which enhanced levels of a particular wall polysaccharide are desirable. Enforced by the limited availability of genes involved in cell wall biosynthesis, at the start of this thesis work, this approach was examined for only two genes. Nevertheless, more and more genes involved in cell wall biosynthesis are being identified enabling future experiments to knock out or enhance specific elements in the biosynthetic machinery. A recently identified putative glycosyltransferase required for normal pectin synthesis in *Arabidopsis* (Bouton *et al*., 2002) would for instance be an interesting candidate to examine the possibility of down or up-regulating pectin levels by genetic modification. Our results described in chapters 3, 4 and 5 already show that the potato tuber cell wall is amenable to genetic modification, and that there is a wide range of wall modifications which is tolerated by the tubers.

The increased levels of cell wall bound galactan in potato tubers with increased *StUGE45* and *StUGE51* expression (Chapter 5) indicates the importance of UDP-Gal levels for galactan deposition in the cell wall. The knowledge on the biosynthetic pathway of the different UDP-sugars involved in cell wall biosynthesis and the

availability of more and more of the sugar converting enzymes may enable the regulation of also other UDP-sugar levels (increasing as well as decreasing). However, as postulated in the UGE potato transformants, alterations of one enzyme and subsequently the pool size of one or two UDP-sugars may also effect other UDP-sugar levels. When aiming for a specific modification these secondary effects, which may be desirable as well as undesirable, will have to be taken into account.

The cellulose synthase transformants (described in chapter 3 and 4) showed the possibility of altering cellulose levels by modulating the expression of the *CesA* genes. The plant *CesA* genes, homologous to bacterial cellulose synthases (Delmer 1999), are generally believed to be part of the so-called rosette terminal complex (TC) which was first described in plants by Mueller and Brown (1980). One rosette is suggested to be composed of six elementary particles, each consisting of probably six *CesA* proteins which all polymerise a glucan chain, able to together synthesise a cellulose microfibril composed of 36 glucan chains (6 elementary particles times 6 polymerising *CesA* units). Even though, the exact composition is still unclear, the TCs are suggested to contain also a sitosterol-β-glucoside transferase, and an endo-glucanase (Peng *et al.*, 2002; Read and Bacic, 2002). While the effect of decreased cellulose upon knock out of *CesA* genes (either by mutation or genetic modification) was also found in other plants an example of increased cellulose levels by increased *CesA* expression levels was not shown before. This is in contrast with the general believe that more (different) *CesA* proteins must be present in one cellulose synthase complex (Desprez *et al.*, 2002; Fagard *et al.*, 2000; Taylor *et al.*, 2000) to generate cellulose. This may indicate that the *StCesA14* is able to form a homomeric cellulose synthase complex (in which no other *StCesA* proteins are present) or that WT expression levels of this *CesA* gene are a limiting factor in the formation of complexes in which the *StCesA14* is taking part. Nevertheless, these plants show that using genes involved in wall polysaccharide backbone polymerisation can be successful, which is promising in respect to future experiments to directly increase levels of for instance pectin. Additionally the introduction of a construct expressing a homologous *CesA* gene may be useful in studies unravelling the cellulose synthase complex. Taylor and co-workers (2000) introduced a tagged *AtCesA7* in an *Arabidopsis* mutant with a dysfunctional *CesA7* gene. Affinity purification of the corresponding protein showed it to co-purify with the *AtCesA8* gene product suggesting that these two proteins are linked in an *in vivo* situation. Similar studies in potato with the identified potato *CesA* clones may reveal which *StCesA* gene products are present in the same complex and possibly also enable the isolation of other components of the cellulose synthase complex.

The antisense cellulose synthase plants described in chapter 4 show that the down-regulation of individual members of the *CesA* gene family is also possible. To achieve this the class specific region (CSR) was used for antisense expression. This region is only present in plants, and is believed to determine the genetic difference between the varying *CesA* genes in one plant (Vergara and Carpita, 2001). These results showed that is possible to specifically down regulate one *CesA* gene even though they are part

of a large multi-gene family. An experiment in *Arabidopsis* in which other and larger regions of these genes were used in antisense constructs also showed a down-regulated expression of the corresponding gene only (Burn *et al.*, 2002).

The discovery of new genes involved in wall polysaccharide synthesis
To date, the availability of genes involved in cell wall biosynthesis is still limited and a major disadvantage when examining cell wall modification by interfering with the biosynthetic machinery. The identification of new genes functioning in cell wall biosynthesis will thus be a determining factor in designing future experiments. In addition, the final determination of the correlation between structural characteristics and the presence of a particular gene or its transcription level may be used in breeding programs to select for desirable cell wall characteristics using molecular markers. Unfortunately, the study to isolate new genes by RNA fingerprinting in a potato leaf protoplast system (Chapter 6) was not successful. This experiment demonstrated the limitations when studying a process like primary cell wall biosynthesis. In contrast to some other processes (like secondary cell wall biosynthesis) it is difficult to obtain a system in which synthesis is occurring in a synchronised and controlled fashion. It thus seems likely that a better understanding of the molecular processes controlling cell wall biosynthesis will come from other studies as for instance the identification of mutants. The recent identification of a putative glycosyltransferase required for normal pectin synthesis in the *Arabidopsis* mutant *QUASIMODO1* shows the relevance of mutagenesis experiments in the identification of cell wall synthases (Bouton *et al.*, 2002). A subsequent isolation of other plant homologs of this gene and the generation of transformants with different expression levels of the gene will aid in determining the exact function of a gene. The final identification of more and more genes involved in cell wall biosynthesis and modification will also enable new RNA fingerprinting studies focussed on the importance of these known genes for specific tissues or developmental stages. Both these approaches will increase the understanding of processes involved in cell wall biosynthesis and the relevance of the whole range of cell wall structures showing a tissue and time specific localisation.

STUDYING ALTERED CELL WALL POLYSACCHARIDES TO DETERMINE THEIR BIOLOGICAL IMPORTANCE

Specific RG I modifications show structural importance of its components
The successful modification of potato wall pectin structure by the introduction of RG I degrading enzymes resulted in plants which can be very useful in assigning putative functions to the individual RG I components. The comparison of the *e*GAL (Sørensen *et al.*, 2000), *e*GARA (Skjøt *et al.*, 2002) and *e*RGL (Chapter 2) transformants shows the importance of the complete RG I backbone compared to its individual galactan and arabinan side-chains. The histological and morphological changes in the *e*RGL

transformants suggest an important role of the RG I backbone for the integrity and function of the wall, at least in potato tubers.

Several studies on developmental and tissue dependent localisation of RG I attached galactan suggest that galactan is present mostly in older cells and is positively correlated with firmness (Bush et al., 2001; Jones et al., 1997; McCartney et al., 2000; Redgwell et al., 1997; Vicré et al., 1998; Willats et al., 1999). However, in the eGAL transformants, with a reduction of RG I galactan hairs, there were no indications of altered tuber firmness, development or morphology. Combination of the results from the eGAL potatoes with the observations of pea and fruit ripening suggests that removal of the galactan chains does not directly reduce the strength of the cell wall (see Oomen *et al.* (2003) for a more detailed discussion). It is likely that the loss of galactan chains enables other enzymes to access regions of the cell wall to modify other cell wall components. This is in line with the observations by Foster et al. (1998) and Fenwick et al. (1999), who suggested that galactan hairs might be important for decreasing the pore size of cell walls and hence play a role in wall porosity.

No particular function has been suggested to date for the RG I arabinan hairs, even though they are developmentally and spatially regulated (Bush et al., 2001; Orfila and Knox, 2000; Orfila et al., 2001; Willats et al., 1999; Willats et al., 2001a). The eGARA transformants (Skjøt *et al.*, 2002) are comparable with the *Arabidopsis mur4* mutant (Burget and Reiter, 1999) which also shows a reduction in L-arabinose (respectively 69 and 50%). These plants do not show any phenotypic defect and the reduction in L-arabinose does not seem to be compensated for by another specific monosaccharide. This suggests that a reduced level of RG I bound arabinan does not interfere with normal plant development, even though the phenotype of the plants at other environmental and physiological conditions remains to be studied.

The altered histology and morphology of potato tubers expressing the eRGL (Chapter 2) suggests an important function of the RG I backbone structure, since the removal of only the galactan and arabinan hairs did not result in cytological differences. The altered tuber morphology in the eRGL plants, caused by changes in the periderm and cortex, are probably the result of random divisions and abnormal expansions in these tissues. These results suggest that the RG I is necessary for normal periderm development even though the underlying reason is not yet clear. A similar effect was found in the *Arabidopsis emb30* mutants (Shevell et al., 2000). These mutants show a localization of pectic polysaccharides at cell corners and interstitial spaces together with the normal wild type localization in the cell wall. The authors hypothesize that the abnormal localization, which is likely to be caused by a failure in the deposition of the pectic polysaccharides, might interfere with normal cell division and disturb the cellular polarity. The abnormalities in the eRGL and *emb30* plants appear to be of a similar kind. However, it still has to be established whether or not the *emb30* mutation has affected the RG I structure.

As said the different pectin transformants were so-far only studied during normal environmental conditions. In several transformants phenotypical abnormalities were

only identified upon growth at restrictive conditions. A more detailed analysis of the *e*RGL, *e*Gal and *e*GARA transformants at different developmental stages and environmental conditions may provide new insights on the effect of the respective modifications and significance of wall polysaccharides. Nevertheless, the identified alterations in these pectin transformants showed the importance of the RG I backbone for fixation of the complete polysaccharide, including the galactan and arabinan side-chains, in the cell wall, and normal plant development. These observations are a useful contribution to other studies for a further understanding of the pectin polysaccharides, as deposited in the plant cell wall (Vincken *et al*., 2003).

Even though, tubers of the UDP-Glc-4-epimerase transformants show increased levels of cell wall bound galactan it is not yet clear which cell wall polysaccharides have been affected by this modification. An increased level of RG I bound galactan would be very interesting and useful to study the hypothesis that the galactan side chains are mostly involved in determining the pore size of the cell wall. Differences in pore size between these transformants and WT plants may for instance be studied by uptake of e.g. inert gold particles (O'Driscoll *et al*., 1993; Schaffer and Wisniewski, 1987) or labelled dextrans of known size (Baron-Epel *et al*., 1988). Another method is by studying the penetration of probes of high osmolarity resulting in plasmolysis of cell contents (Carpita *et al*., 1979). As found in many studies, fruit ripening is often related with the degradation of RG I bound galactan by e.g. β-galactosidases and exo-galactanases (reviewed by Brummel and Harpster, 2001). Increased levels of galactan may slow down this process and subsequently also delay the process of fruit ripening. Our epimerase transformants have proven the relevance of UDP-galactose levels for final levels of galactan in the cell wall. It may thus be expected that antisense expression of the potato *UGEs* results in decreased levels of cell wall bound galactan. This can affect the composition of xyloglucan, galactomannan or RG I, all containing galactosyl residues. The latter will probably give a similar effect as the plants from Sørensen and co-workers (2000) expressing the *endo*-galactanase. One major difference between these plants would be the time-point at which the modification is induced. It is possible that by limiting the synthesis and attachment of galactosyl residues or galactan chains to RG I a compensatory or alternative structure is synthesised which was not observed in the *e*Gal transformants. The initially less branched RG I backbone may for instance be more receptive to attachment of more arabinan side-chains if both UDP-arabinose as well as the necessary enzymes are available.

Tolerance of cellulose depletion shows to be plant and tissue specific
Regarding the generally accepted importance of cellulose for strength in the cell wall it is remarkable that antisense potato tubers do not show any obvious phenotypic alteration. In other plants, like *Arabidopsis* and tobacco, moderate reductions of cellulose already resulted in phenotypical alterations such as an effect of disturbed cellular development (Arioli *et al*., 1998; Burn *et al*., 2002; Burton *et al*., 2002; Fagard

et al., 2000; His *et al.*, 2001). However, it must be noted that most of these mutants only revealed a phenotype when grown at restrictive conditions. Comparative experiments have not yet been performed for the potato cellulose synthase transformants, which were only grown at normal *in vitro* and greenhouse conditions. The difficulty of generating antisense *CesA* potato plants with the constitutive CaMV promoter additionally suggests that cellulose depletion of other potato plant tissues or organs severely affects their development. The ability of these transformants to resist a reduction of cellulose (to 50% of WT levels) in the tubers thus seems to be a tissue specific event. The fact that not all potato transformants with decreased cellulose levels show modifications in their pectin composition indicates a delicate balance between cellulose and pectin biosynthesis. Nevertheless, even though modifications in pectin composition do not seem to be necessary for a compensation of the depleted cellulose, some transformants show altered levels of pectin. In the csr10 and 17 transformants (Chapter 4) increased uronic acid levels and a decrease in galactan is correlated with the decreases in cellulose. This may suggest that potato tubers can withstand a larger reduction in cellulose than other plants or plant parts. The accompanying alterations in pectin levels may not be appearing solely for compensating loss of strength upon depletion of the cellulose/xyloglucan network as suggested in many other plant tissues and systems.

In addition, also increased levels of cellulose resulted in modifications of the pectin structure. One explanation may be that the excessive use of UDP-Glc for cellulose biosynthesis has resulted in shifts of other UDP-sugar levels giving reduced levels of galactose and increased uronic acid levels. Again, this indicates that not all changes in cell wall polysaccharide structures and their abundance are a direct effect of compensatory mechanism but may simply be imposed by shifts in biosynthetic pathways. This has to be taken into account when assigning a specific biological function to particular observations in transformants.

APPLICATIONS OF TAILOR MADE CELL WALL POLYSACCHARIDE STRUCTURES

Even though, cell wall preparations from the described transformants have not yet been examined for any particular application, the initial results indicate the feasibility to generate plants with specific cell wall composites. This was most obvious in the modifications of the RG I structure obtained by the introduction of RG I degrading enzymes from *A. aculeatus*. As said, the potato pectin should be preferably less branched and highly methylated to function as a gelling agent, stabiliser or emulsifier. A complete degradation of the RG I molecule showed to negatively affect tissue development in the tubers, which are subsequently not very suitable for starch or other industries. Nevertheless, the individual degradation of the galactan and arabinan side chains did not result in any obvious phenotypic alterations. Plants in which these

characteristics are combined may be interesting to test on an application level. Further, Willats and co-workers (2001b) showed that both the pattern as well as the degree of methyl-esterification of homogalacturonan domains are determining factors in influencing the compressive strength, elasticity, water holding capacity and the porosity of calcium-mediated pectin gels. The presence of HG with blockwise as well as non-blockwise distribution of methyl-esters at specific localisations in plant tissues and cells indicates the importance of the gelling characteristics also for *in vivo* purposes. The availability of many fungal and plant pectin methyl esterases (PMEs), with a different mode of action, enables their use in new studies for targeted *in muro* modifications of pectin structures. A study in which a *Petunia* PME was over-expressed in potato plants resulted in enhanced stem elongation and a reduction in tuber yield (Pilling *et al*., 2000). However, an obvious modification in pectin structure, leading to these physiological alterations, was not identified. A more detailed (antibody studies) analysis of these plants and the generation of transformants expressing other PMEs will be interesting to study the relevance of different variations of HG esterification and may additionally alter the industrial applicability of potato pectin when these modifications result in improved gelling characteristics.

Another useful modification is the increased level of cellulose in the potato plants overexpressing the *StCesA14* (Chapter 3). Even though an increase in cellulose levels is probably not very useful in potato plants it may well find an application in other crop plants as for instance flax. Currently, cotton is the major natural source for textile fibres because of its high content of highly crystalline, nearly pure cellulose. However, with the increasing knowledge on cell wall biosynthesis, and the genes involved, studies focussed on generating crops with an increased fibre quality are getting more interest. As described by Ebskamp (2002), the applicability of cell wall fibres from for instance flax and hemp may improve upon *in planta* modification of the cell wall polysaccharide structures. An increased cellulose content in this fibre fraction may directly increase the applicability or may indirectly change the characteristics of the other polysaccharides and for extractability of the fibre bundles. The possibility to further increase the cellulose content in cotton by enhanced *CesA* expression levels is of course also very interesting.

FINAL REMARKS

Even though in the past 5 years a lot of progress has been made on studying the biological significance of the individual cell wall polysaccharides and their biosynthetic machinery, many questions are still not answered. The transformants described in this thesis describe the feasibility of generating specific *in muro* alterations of cell wall polysaccharides and their applicability in answering some of the questions on the importance of the polysaccharide structures. The generation of new transformants in the future will definitely aid in a further understanding of the plant

cell wall. Some of these transformants can be generated with the currently available tools for instance by generating transformants in which introduced, increased or decreased expression levels of the already examined genes are combined. To enable the generation of more transformants in the future a significant input will come with the identification of new cell wall related genes. Most likely several of these will be identified in mutagenised plants with a dysfunctional gene. A further analysis of the gene function can subsequently be obtained by isolating the gene homologue in other plant species and its use for the generation of transgenic plants with up and down regulated expression levels. Additionally a detailed understanding of the correlation between a specific gene, its transcription level and the final structure of a particular cell wall polysaccharide may be used in breeding programs (by using molecular markers) to generate new plant lines with particular cell wall characteristics.

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Summary

Apart from its well known uses in the human diet a large amount of the grown potatoes (about one third in the Netherlands) is used for the isolation of starch which is used in several food and non-food applications. The cell wall fibres comprise a large portion of the waste material remaining after the starch isolation process. While cell wall fibres from some other plant species are used in food and non-food industry, a structural alteration of the potato fibres is necessary before similar applications are possible. However, before it is possible to generate plants with a tailor-made cell wall composition, questions concerning the best approach have to be answered and the necessary tools will have to be identified and made available. This thesis describes the results of a study investigating the possibilities to generate transgenically modified potato plants with an altered cell wall composition. These experiments were mostly focussed on altering pectin composition. This is particularly interesting because several studies already showed that many different pectin structures occur in specific plants, plant tissues and developmental stages. Plants with a specific alteration in pectin structure may aid in revealing the biological significance of these different structures. Additionally, the possibility to produce a particular pectin structure may be useful for the food industry, in which pectins from other plant species are already used as a gelling agent.

At the start of the work described in this thesis only few genes involved in cell wall biosynthesis were identified which favoured the heterologous expression of fungal pectin degrading enzymes. A rhamnogalacturonan lyase (*e*RGL) from *Aspergillus aculeatus*, which is able to cleave the rhamnogalacturonan I (RG I) at specific sites, was introduced. The *e*RGL was successfully expressed (under control of the granule-bound starch synthase promoter) and translated into an active protein, demonstrated by *e*RGL activity in the tuber extracts. These tubers showed clear morphological alterations, including radial swelling of the periderm cells and development of intercellular spaces in the cortex. Sugar compositional analysis and antibody labelling studies showed a large reduction in galactan and arabinan side-chains of RG I. These data show the possibility of specifically modifying cell wall polysaccharide structures by the introduction of such a pectin degrading enzyme. Additionally, the results suggest that RG I has an important role in anchoring galactans and arabinans at particular regions in the wall and in normal development of the periderm. The utility of these transgenic plants in answering questions concerning the biological importance of cell wall polysaccharides is evident.

Apart from modifying the cell wall composition by the introduction of pectin degrading enzymes two experiments were performed focussed on interference with the biosynthetic machinery of the plant cell wall at different levels. The first study concerns the modulation of cellulose synthase (*CesA*) gene expression. Since this enzyme is polymerising the β-1,4 glucan chains forming cellulose, its altered expression is likely to directly affect the level of cellulose in the wall. In the second study the expression of the UDP-Glc-4-epimerase (*UGE*) was modulated. The *UGE* is

responsible for the conversion of UDP-glucose to UDP-galactose and vice-versa. Its altered expression is likely to affect the amount of cell wall bound galactan.

Four *CesA* genes were isolated from potato and one full length cDNA clone was used for up- and down-regulation of the corresponding RNA expression levels controlled by the granule-bound starch synthase promoter. Fourier Transform Infra-Red microspectroscopy (FTIR) was used for the identification of transformants with altered levels of cellulose in their tuber cell walls in comparison to WT plants. A further quantification of these results, by measuring the cellulose content in the cell wall material, showed that by modulating the *CesA* expression levels, tubers with levels of cellulose ranging from 50 to 200% of the WT amount were obtained. Especially the increase in cellulose is quite remarkable and in contradiction with the general believe that expression of more than one *CesA* gene (and possibly even more genes) is necessary to achieve such a modification. By using a specific region of the other three *CesA* genes in antisense experiments we managed to individually down regulate these genes and concomitantly the cellulose levels in the tubers of these plants. The use of this so-called class specific region (CSR), which is only present in plant cellulose synthases and is believed to determine the genetic difference between the different *CesA* genes in one plant, showed to be sufficient to down regulate the corresponding gene. In contrast to many other plants and plant systems, depletion of cellulose (to 50% of WT level) in potato tubers did not result in any phenotypic alterations. However, our potato plants were grown at normal conditions while some of the cellulose synthase mutants only revealed a phenotype when grown at restrictive conditions. Another important result is the fact that not all potato transformants with decreased cellulose levels show modifications in their pectin composition. This indicates a delicate balance between cellulose and pectin levels and that an altered pectin composition in plants with depleted cellulose is not necessarily a response upon reduced strength of the cell wall.

For the UDP-Glc-4-epimerase two potato cDNA clones (*StUGE45* and *StUGE51*) were identified and used for overexpression in potato tubers. The increased levels of cell wall bound galactan in these tubers indicates the importance of UDP-galactose levels for galactan deposition in the cell wall. Additionally these plants showed a small decrease in the amount of galacturonan. This suggests that alterations in the UDP-galactose pool size can influence the levels of nucleotide sugars which are used for the synthesis of other polysaccharides. Additionally, the elevated expression levels of the two *UGE*s showed to have different effects, which suggests that they have a different function in plant development. Further research has to show whether other polysaccharides than cell wall galactan are affected by this decrease in galactose. Xyloglucan and galactomannan also contain galactosyl residues and the decrease does not necessarily affect the levels of RG I bound galactan.

Both these studies show the possibility to induce alterations in the cell wall composition by interfering with the biosynthetic machinery. Identification of more genes involved in cell wall biosynthesis is necessary to enable new studies in the

future. An RNA fingerprinting experiment was performed to investigate the possibility of identifying new genes involved in primary cell wall biosynthesis. Potato leaf protoplasts showed to regenerate a new cell wall in the first 18h after transfer to a culture medium. At 5 distinct time-points RNA was isolated and the expressed genes were visualised using cDNA-AFLP. Around 8500 transcript derived fragments (TDFs) were visualised from which 156 were isolated and sequenced. However, no cell wall related TDFs were identified. This indicates that even though the protoplasts actively regenerate a new cell wall, this did not result in highly increased expression of genes involved in cell wall biosynthesis or modification.

In summary the experiments described in this thesis showed that different approaches can be used to generate a modified cell wall composition in potato tubers. These genetically modified plants have shown to be an interesting study material for unravelling the biological function of different cell wall polysaccharide structures. Additionally these transformants obviously showed that the potato tuber cell wall is amenable to genetic modification. There is a wide range of wall modifications which is tolerated by the tubers, which may hold a promise for the future in valorising the fibre fraction of potato after starch isolation.

Samenvatting

Hoewel de aardappel over het algemeen enkel als direct consumptiemiddel (gewoon gekookt of verder verwerkt tot bijvoorbeeld friet of chips) gezien wordt zijn er ook andere toepassingen mogelijk. Maar liefst een derde van de in Nederland geteelde aardappels is bestemd voor de zetmeelindustrie en wordt gebruikt voor toepassingen in onder andere de papier- en textiel-industrie. Het celwandmateriaal, dat als afval overblijft na de isolatie van het zetmeel, heeft een laagwaardige toepassing als toevoeging aan veevoer. Dit is in tegenstelling tot pectine polymeren (één van de celwand componenten) van citrusvruchten die veelvuldig gebruikt worden in de levensmiddelenindustrie (bijvoorbeeld als geleermiddel in jams). De sterk vertakte structuur van aardappel pectine is minder geschikt hiervoor. De ontwikkeling van planten met een gemodificeerde celwandsamenstelling zou kunnen resulteren in nieuwe industriële toepassingen van aardappel celwandmateriaal. Voordat men echter zover is, dienen nog vele vragen betreffende de functie, alsmede de synthese en afzetting van de verschillende celwandcomponenten, beantwoord te worden.

In dit proefschrift worden verschillende experimenten beschreven gericht op de bestudering van de mogelijkheden om aardappels met een gemodificeerde celwandsamenstelling te genereren. Hierbij lag vooral de nadruk op het modificeren van het pectine in de celwand. Meerdere studies hebben al aangetoond dat verschillende pectine structuren voorkomen afhankelijk van de plant soort, het plant weefsel en het ontwikkelingsstadium van de plant. Planten met een specifieke verandering (bijvoorbeeld door middel van genetische modificatie) van de pectine structuur kunnen mogelijk een bijdrage leveren aan het ophelderen van de biologische functie van deze verschillende pectine structuren.

Bij aanvang van dit onderzoek waren er slechts een beperkt aantal celwandbiosynthese genen bekend. Daarom werd een eerste experiment opgezet waarin een celwand afbrekend schimmelenzym in de aardappelplant werd geïntroduceerd. Een rhamnogalacturonaan lyase (*e*RGL) van *Aspergillus aculeatus*, dat in staat is om het rhamnogalacturonaan I (RG I, een pectine hoofdpolymeer dat op verschillende plaatsen verschillende typen zijketens kan hebben) op specifieke plaatsen kan knippen, werd in de aardappel tot expressie gebracht. De aardappels van deze planten hebben een onregelmatige buitenkant en laten op microscopisch niveau zien dat de periderm cellen zijn opgezwollen en dat het weefsel minder gestructureerd is. Bovendien zijn er meer en grotere intercellulaire ruimtes aanwezig in het cortex weefsel. De analyse van de suikersamenstelling en de bestudering van knolweefsel met specifieke antilichamen toonden een sterke afname in de galactaan en arabinaan zijketens van het RG I. Deze data bevestigen dat het mogelijk is om de structuur van celwand polysacchariden specifiek te wijzigen door middel van de introductie van een pectine afbrekend enzym. Bovendien tonen deze planten aan dat het RG I een belangrijke rol speelt bij de verankering van de galactaan en arabinaan zijketens op bepaalde plaatsen in de celwand en het belang van RG I voor een normale ontwikkeling van het periderm. Planten met dit soort modificaties kunnen dus een bijdrage leveren aan de opheldering van de biologische functie van bepaalde celwand polysacchariden.

Naast modificatie door middel van de introductie van pectine afbrekende enzymen zijn ook twee studies uitgevoerd gericht op het beïnvloeden van de synthese van verschillende celwand polysachariden. De eerste van deze twee experimenten is gericht op modulatie van de expressie van het cellulose synthase (*CesA*). Dit enzym is verantwoordelijk voor de polymerisatie van de β-1,4 glucaan ketens die uiteindelijk de cellulose fibrillen vormen. Een veranderde expressie van dit gen beïnvloedt waarschijnlijk direct de hoeveelheid cellulose in de celwand. In het andere experiment is de expressie van het UDP-Glc-4-epimerae (*UGE*) veranderd. Het *UGE* is verantwoordelijk voor de conversie van UDP-glucose tot UDP-galactose en omgekeerd. De gemodificeerde expressie niveaus van de twee *UGE* genen resulteren zeer waarschijnlijk in een verandering van de hoeveelheid celwandgebonden galactaan. Vier *CesA* cDNA klonen, waarvan één met de volledige cDNA sequentie, werden geïsoleerd uit een aardappel cDNA bank. De volledige kloon werd gebruikt om de RNA expressie van het betreffende gen in verschillende experimenten te verhogen dan wel verlagen. Fourier Transform Infra-Red microspectroscopie (FTIR) werd gebruikt voor de identificatie van die transformanten die een cellulosegehalte hadden dat afwijkt van het niveau in wild type planten. Een verdere kwantificering van deze resultaten waarbij het cellulosegehalte in het celwandmateriaal werd gemeten toonde aan dat door deze modulatie van de *CesA* RNA expressie niveaus, aardappels met een cellulosegehalte van 50 tot 200% van het wild type niveau verkregen waren. Vooral deze toename in cellulose is opmerkelijk vanwege het algemeen geaccepteerde idee dat meer dan één *CesA* gen (en waarschijnlijk ook nog andere genen) van belang zijn voor de synthese van cellulose. Een ander experiment met deze cellulose synthase genen betrof de specifieke verlaging van de, RNA expressie van, drie van de aardappel genen. Hiervoor werd de zogenaamde 'class-specific-region' (CSR) in antisense oriëntatie in de knol tot expressie gebracht. Deze 'class-specific-region' is alleen aanwezig in cellulose synthase genen van planten en lijkt van groot belang te zijn voor het bepalen van de diversiteit tussen de verschillende *CesA* genen. In het uitgevoerde experiment bleek dat de antisense expressie van enkel de CSR voldoende is voor het verkrijgen van een specifieke verlaging van de RNA expressie van het betreffende *CesA* gen. In tegenstelling tot resultaten in vele andere planten resulteerde de afname van cellulose (tot 50% van wild type niveau) in aardappelknollen niet tot fenotypische afwijkingen. Hierbij moet echter opgemerkt worden dat de betreffende aardappelplanten alleen nog maar geteeld zijn bij standaard omstandigheden terwijl verschillende cellulose synthase mutaties of modificaties in andere planten ook enkel een afwijkend fenotype toonden bij afwijkende condities. Een ander opmerkelijk resultaat is het feit dat niet alle transformanten met een afname in het cellulosegehalte een verandering in de samenstelling van het pectine laten zien. Dit suggereert een delicate balans tussen de cellulose en pectine samenstellingen en dat een veranderde pectine samenstelling in planten met een cellulose deficiëntie niet noodzakelijkerwijs een respons is op een afname in de stevigheid van de celwand.

In het UDP-Glc-4-epimerase experiment werden twee aardappel cDNA klonen (*StUGE45* en *StUGE51*) geïsoleerd die beide gebruikt werden voor overexpressie van de betreffende genen in de aardappelplant. De transgene aardappelknollen toonden inderdaad een toename in de hoeveelheid celwand gebonden galactaan wat duidelijk het belang van de hoeveelheid UDP-galactose voor de uiteindelijke afzetting van galactaan in de celwand aangeeft. Bovendien toonden deze planten nog een lichte afname in de hoeveelheid galacturonzuur. Dit suggereert dat veranderingen in de hoeveelheid UDP-galactose ook invloed hebben op hoeveelheden van andere UDP-suikers die van belang zijn voor de synthese van andere polysacchariden. Bovendien, bleek de verhoogde expressie van de twee *UGE*s een verschillend effect te hebben. Dit suggereert dat beide genen een verschillende functie hebben bij de ontwikkeling van de plant. Verder onderzoek zal moeten uitwijzen welke andere polysacchariden dan celwand galactaan zijn beïnvloed door deze afname in galactose. Ook xyloglucaan en galactomannaan bevatten galactosyl residuen en de afname van UDP-galactose heeft niet noodzakelijkerwijs effect op de hoeveelheid RG I gebonden galactaan.

De hierboven genoemde studies tonen de mogelijkheid om de celwand samenstelling te modificeren door het introduceren van nieuwe genen of door het beïnvloeden van gen expressie niveaus van endogene genen die betrokken zijn bij de celwandbiosynthese. Hoewel meerdere celwandafbrekende enzymen (en de bijbehorende genen) voorhanden zijn is het voor toekomstige experimenten van belang dat er meer genen die betrokken zijn bij de celwandbiosynthese geïsoleerd worden. Om de mogelijkheid tot het isoleren van nieuwe genen, betrokken bij de synthese van de primaire celwand, te bestuderen is een RNA-fingerprinting experiment uitgevoerd. Protoplasten uit bladmateriaal van aardappelplanten genereren een nieuwe celwand in de eerste 18 uur na het overbrengen naar een cultuur medium. Op vijf specifieke tijdstippen werd RNA geïsoleerd en de verschillende expressie patronen werden gevisualiseerd met behulp van cDNA-AFLP. Ongeveer 8500 'transcript derived fragments' werden gevisualiseerd waarvan er 156 geïsoleerd werden om vervolgens de DNA sequentie hiervan te bepalen. Er werden geen bekende celwand gerelateerde genen geïdentificeerd. Dit geeft waarschijnlijk aan dat hoewel de protoplasten actief een nieuwe celwand regenereren dit niet noodzakelijkerwijs bewerkstelligd wordt door een duidelijke toename in expressie van genen betrokken bij de biosynthese of modificatie van de celwand.

Samengevat beschrijft dit proefschrift verschillende mogelijkheden om de celwand samenstelling van aardappelknollen te modificeren en het belang van deze planten voor het bestuderen van de biologische functie van de verschillende celwand polysacchariden. De planten tonen duidelijk de tolerantie van de aardappel voor dit soort celwand modificaties, wat suggereert dat het ontwikkelen van aardappels waarvan na isolatie van het zetmeel nog een bruikbaar en beter geschikt polysaccharide overblijft in de toekomst zeker tot de mogelijkheden behoort.

Nawoord

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CURRICULUM VITAE

Op 23 december 1974 werd ik (Ronald Oomen) geboren in Etten-Leur. Na het VWO aan de Katholieke Scholengemeenschap Etten-Leur ben ik in 1993 begonnen met de medische laboratorium opleiding aan de Hogeschool West-Brabant. Daar koos ik voor de specialisatie biotechnologie. Mijn stage heb ik gedaan bij het Laboratoire Français du Fractionnement et des Biotechnologies in Lille, Frankrijk. Voor mijn afstudeerproject koos ik vervolgens het laboratorium voor Plantenveredeling aan de Wageningen Universiteit. Na de afronding van dit afstudeerproject en mijn afstuderen in 1997 bleef ik verbonden aan Plantenveredeling om in September 1997 als AIO te beginnen. Het onderzoek vond plaats binnen het door de EU gefinancierde project; "Remodelling Pectin Structure in Plants" en heeft geleid tot dit proefschrift. Van augustus tot en met november 2002 was ik in het kader van een short-term EMBO fellowship werkzaam aan de University of Newcastle Upon Tyne in Engeland.

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