Targeted and non-targeted effects in cell wall polysaccharides from transgenetically modified potato tubers

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Abstract

The plant cell wall is a chemically complex network composed mainly of polysaccharides. Cell wall polysaccharides surround and protect plant cells and are responsible for the stability and rigidity of plant tissue. Pectin is a major component of primary cell wall and the middle lamella of plants. However, pectin biosynthesis in planta and the mechanisms underlying the influence of structural differences arising from a modified biosynthesis machinery on functional properties remain poorly understood. In our research, the changes in the chemical structures of cell wall polysaccharides after transgenic modification of potato tuber polysaccharides were examined. The cell wall material from potato wild-type varieties, from known and from new potato transgenic lines targeting changes of the homogalacturonan or rhamnogalacturonan I backbone were isolated and characterized. The modified cell wall polysaccharides were examined by determining their individual monosaccharide levels on fresh weight base and their cell wall characteristic parameters, and levels of acetylation and methyl esterification of cell wall pectin. Data for both targeted and non-targeted structures of cell wall polysaccharides from wild-type and transgenic potatoes were obtained. A shorter galactan side chain was found from the buffer soluble pectin and calcium bound pectin of β-galactosidase (β-Gal) transgenic lines. All pectin fractions from rhamnogalacturonan lyase (RGL) transgenic lines had less galactan chains attached to their rhamnogalacturonan I backbones. Two uridine diphosphate-glucose 4-epimerase (UGE) transgenic lines, UGE 45 and UGE 51, had diverse effects on length of the galactan side chain. The xyloglucans from the RGL and UGE transgenic lines retained its XXGG building blocks but differed in the proportion of repeating units compared to the respective wild-type varieties. In contrast, the β-Gal transgenic lines predominantly consisted of XXXG-type xyloglucan in the 4 M alkali extract, but showed XXGG-type building blocks in 1 M alkali extract. In addition, a quick-screening method was validated and used to analyze 31 transgenic lines and their respective wild-type potato varieties. An overall comparison of pectin backbone, pectin side chains, acetylation and methyl-esterification of pectin, pectin content and (hemi)cellulose content of cell wall polysaccharides from these transgenic lines provided a better insight in the frequency, level and combination of both targeted and non-targeted structural changes compared to that of their respective wild-type varieties. The same evaluation method was used to correlate cell wall composition in wild-type and selected transgenic lines and their established gene expression with the texture of corresponding cooked potato cubes. Changed physical properties for the genetically modified tubers could be connected to specific cell wall characteristics. Tubers from transgenic lines containing cell wall pectin with short galactan side chains were less firm after cold processing compared to wild-type tubers. The enhanced understanding of transgenic modifications of potato tubers resulting into significant targeted and non-targeted modifications in cell wall polysaccharides will lead to a better selection of potato lines with tailored cell wall characteristics and desired properties of the tubers during processing.

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Chapter 1

General introduction

Project outline

Plant cell walls are composed of pectin, hemicellulose and cellulose. Pectin, a major component of primary plant cell walls, primarily consists of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). Cell wall polysaccharides are a part of the complex cellular architecture, however, the complete cell wall architecture remains unclear. The Dutch Technology Foundation STW project entitled "Identification of pectin biosynthesis genes in potato and their exploitation to produce tailor-made pectin polysaccharides *in planta*" aims to investigate the structural modification of pectin from transgenically modified potatoes (this thesis) covering both existing and novel lines (Plant Breeding, WUR). The described study aimed to identify parameters to describe polysaccharide characteristics of both cell wall material (CWM) and individual polysaccharide populations from wild-type potato varieties and their transgenic lines.

Potato

In the 16th century, potato (*Solanum tuberosum* L.) was introduced into Europe from South America and since then, this plant has become widely cultivated throughout the world. At present, it is the world's fourth most important food crop after wheat, maize and rice (Food and Agriculture Organization of the United Nations, FAOSTAT; http://apps.fao.org). The popularity of potatoes is attributed to their high crop yield, nutritional value and various culinary uses.

The texture of potato tubers is a key quality determinant of cooked potatoes and determines consumers preference (McGregor, 2007). Tuber texture is correlated with the physical properties of potato cell wall polysaccharides and is affected by processing procedures such as blanching, peeling and cooking (Waldron et al., 2003; Thybo et al., 2006). The effect of cell wall polysaccharides on texture properties is discussed later.

Cell wall

In plants, the cell wall is an important structure that determines the cell shape, connect cells together, provides essential mechanical strength and rigidity and acts as a critical barrier against pathogens (Cosgrove, 2000).

Pectic polysaccharides have been suggested to form a matrix in the primary cell wall via Ca^{2+} cross-links with HG stretches and via boron cross-links with rhamnogalacturonan II (RG-II) (Kobayashi et al., 1999). A second network in the primary cell wall is the cellulose/xyloglucan network. Both networks are reported to act independently within the cell wall (McCann & Roberts, 1991). A schematic representation of the cell wall of parenchyma tissue is shown in Figure 1.1. The pectin network has structural and

interactional linkage functions related to the sizes of pores in the cell wall (McCann & Roberts, 1991). Xyloglucan binds tightly to the surface of the cellulose microfibrils, thereby bridging the space between the microfibrils. The cellulose/xyloglucan network is embedded in a matrix of pectic polysaccharides (Carpita & Gibeaut, 1993).
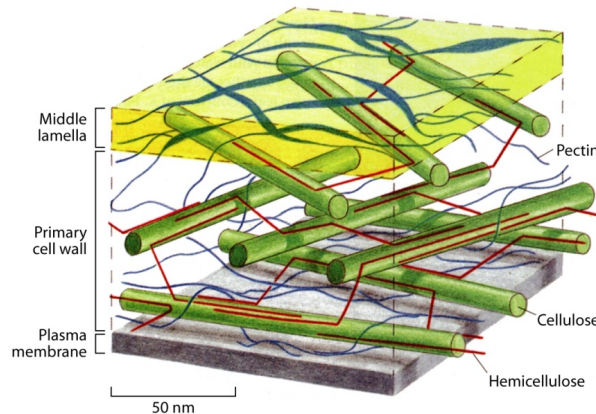
Figure 1.1. Schematic representation of the cell wall of onion parenchyma cells (McCann & Roberts, 1991).
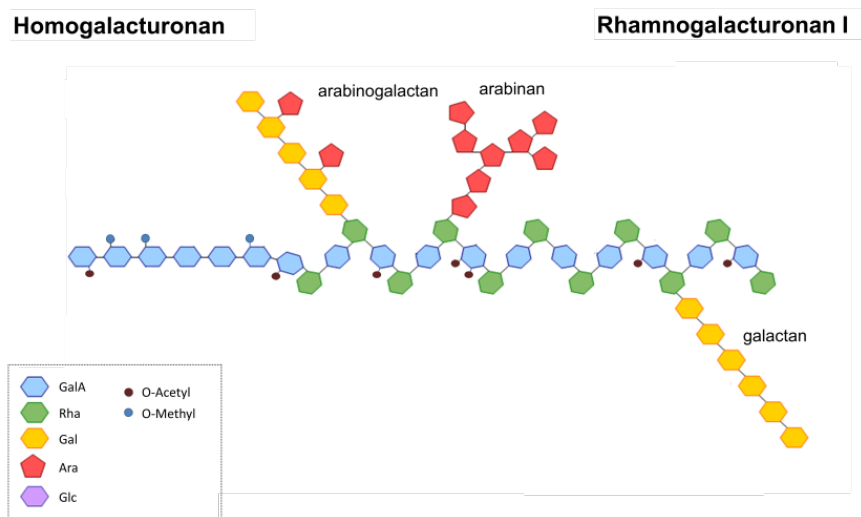
Potato Cell wall

The parenchyma cells of potato tubers have thin, non-lignified primary cell walls (Jarvis et al., 1981). The cell walls are accounts for approximately 1% of potato tubers (Bush et al., 2001). The small amount of cell walls are considered as a distinguishing characteristic of plant's essential elements for their survival (Caffall & Mohnen, 2009). The potato polysaccharides consist mainly of pectic polysaccharides (56%), xyloglucan (11%) and cellulose (30%) and minor proportions of heteromannans (3%) and heteroxylans (<3%) (Vincken et al., 2000).

Pectin

Pectin is a major component of the primary plant cell wall. The molecular structure of pectic polymers influences plant growth and development (Ridley et al., 2001). Pectic polysaccharides include HG, RG-I, RG-II and xylogalacturonan structural elements (Mohnen, 2008). Potato pectin consist of HG (approximately 14% of cell wall polysaccharides) and RG-I (approximately 6% of cell wall polysaccharides) (Vincken et al., 2000). The RG-II and xylogalacturonan are present in minor proportions within potato pectic polysaccharides. In this chapter, we will discuss HG and RG-I since these are the predominant pectin structural elements, present in potato CWM.

Homogalacturonan (HG): In potato, the HG domain comprises approximately 20% of the total pectin polysaccharides, which proportion is much lower than that in other species (approximately 65%) (Harris, 2009). Potato HG, a linear polymer of α-(1→4)-linked galacturonosyl residues (McNeil et al., 1984) (Figure 1.2), can be substituted with methyl esters at C-6 and/or with acetyl groups at *O*-3 (Ishii, 1997). This is different with HG segments present in most other plant species where acetylation with the HG hardly occur. For sugar beet HG structural elements, it has been described that the acetyl groups are attached to *O*-2 and/or *O*-3 of GalA residues (Ralet et al., 2008).

Rhamnogalacturonan I (RG-I): Potato RG-I accounts for approximately 75% of the total pectic polysaccharides, which is higher than for most other species (approximately 20-35%) (Harris, 2009). The RG-I backbone consists of α-(1→2)-L-rhamnose (Rha)-α-(1→4)-D-galacturonic acid (GalA) dimers in a long chain (McNeil et al., 1980) (Figure 1.2). The galactan and arabinan side chains are composed of (1→4)-β-D-galactose (Gal) and/or (1→5)-L-arabinose (Ara) residues and are attached to the *O*-4 position of Rha of the RG-I backbone (Carpita & Gibeaut, 1993; Schols & Voragen, 1994). The galactan side chains of potato may be substituted with arabinosyl residues and will be indicated as arabinogalactan (Øbro et al., 2004) (Figure 1.2). Potato galactans have predominantly (1→4)-linked β-D-Gal residues present in the backbone, interrupted with small proportions (<0.5%) of (1→3)-linked units (Hinz et al., 2005). RG-I arabinan side chains with substitutions of both Ara or Gal could be present (Øbro et al., 2004). In contrast to potato HG, the acetyl groups are attached to *O*-2 position of GalA residues in potato RG-I (Ishii, 1997). The GalA residues in the potato RG-I segment are supposed not to be methyl esterified (Ross et al., 2011).


Figure 1.2. Schematic representation of potato cell wall polysaccharides (adapted from Cankar et al., 2014).

Xyloglucan

Xyloglucans are the major representatives of the hemicelluloses of dicots and are believed to cross-link cellulose microfibrils (Hayashi, 1989; Whitney et al., 1999; Vierhuis et al., 2001). Xyloglucan represent about 11% of potato cell wall polysaccharides (Vincken et al., 2000). The xyloglucan backbone is a polymeric chain of β-(1→4)-linked D-glucopyranose residues that commonly has branches at the *O*-6 position of the glucose (Glc) moieties, which are typically initiated by an α-D-xylose (Xyl) residue. Some of the Xyl units can be further substituted with Gal, Gal-fucose or Ara. These specific side chains, including the Glc residue, are described using single-letter codes (Fry et al., 1993).

In the commonly used nomenclature, G is used for an unsubstituted β-Glc*p*; X is an α-D-Xyl*p*-(1→6)-β-D-Glc*p*; S and L are α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* and β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p*, respectively; and F is an α-L-Fuc*p*-(1→2)-α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-β-D-Glc*p* (Figure 1.3) (Fry et al., 1993). Plant xyloglucans can be divided into two categories according to their repeating structures: XXGG- and XXXG-type of xyloglucan (Vincken et al., 1997). The XSGG and XLFG building blocks as presented in Figure 1.3 represent the XXGG- and XXXG-type of xyloglucan, respectively. The XXGG-type is the most common repeating unit in the family Solanaceae, which includes potato and tomato (Vincken et al., 1996; Vincken et al., 1997). Potato xyloglucan is mainly built from XXGG, XSGG, XLGG and SLGG building blocks (Vincken et al., 1996).
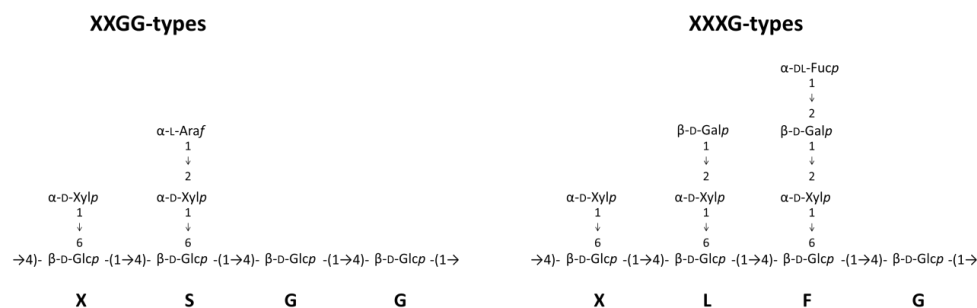
Figure 1.3. Two examples of xyloglucan building blocks. The nomenclature (Fry et al., 1993) indicates the precise side-chain substitution pattern of each β-D-glucosyl residue in the oligosaccharide. XSGG and XLFG are examples of the XXGG- and XXXG-type xyloglucan building blocks, respectively.

Cellulose

Cellulose consists of β-1,4-linked Glc units arranged in long unbranched chains and is one the most abundant macromolecules in nature (Kolpak & Blackwell, 1976). The conformation of cellulose allows the molecules to pack laterally to form microfibrils that

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are stabilized by intra- and inter-molecular hydrogen bonds and van der Waals interactions (Harris & Stone, 2009). Cellulose chain lengths vary across organisms, ranging from approximately 2000 to 20,000 glucose residues. Cellulose microfibrils are the key molecules required for anisotropic growth and for guiding the subsequent assembly of polymers around the cellulose framework in plants (Cosgrove, 1997, 2005). In potato, cellulose accounts for 30% of cell wall polysaccharides (Vincken et al., 2000). The cellulose microfibrils are mostly crystalline and are connected to give an interwoven network of microfibrils in potato cell walls (Harris, 2009).
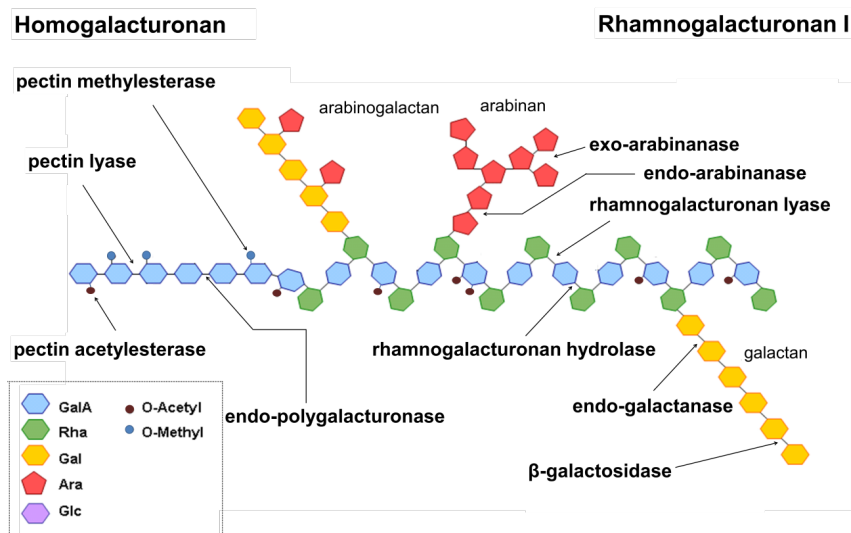
Cell wall-degrading enzymes

Because of the specificity of enzymes, the analysis of digests obtained via the incubation of complex polysaccharides with well-characterized enzymes can provide valuable information on the structure of polysaccharides (Schols et al., 1990). The potential use of enzymatic degradation in the elucidation of the structure of polysaccharides depends on the purity of the enzyme used as well as on its substrate specificity and pattern of action (Voragen et al., 2009). Cell wall-degrading enzymes are described briefly below and the main family numbers of the most important enzymes based on the carbohydrate-active-enzymes database (http://www.cazy.org) are mentioned.

Pectic enzymes

Different types of enzymes are required for the complete hydrolysis of pectin. A schematic representation of the modes of action of enzymes on the pectin backbone is shown in Figure 1.4.

Homogalacturonan-degrading enzymes

Endopolygalacturonase (endo-PG; glycoside hydrolase family 28 (GH 28)) hydrolyzes the α-1,4 glycosidic bonds between two non-esterified GalA residues of the HG region (Benen et al., 1999; Daas et al., 1999) (Figure 1.4). Pectin lyase (PL; polysaccharide lyase family 1 (PL1)) is an endo-acting enzyme that cleaves the linkage between two methyl-esterified GalA residues by β-elimination (Van Alebeek et al., 2002) (Figure 1.4). Pectin methylesterases (PME; carbohydrate esterase family 8 (CE8)) remove methyl esters from the α-(1,4)-linked D-GalA residues of the HG backbone (Figure 1.4). Pectin acetylesterases (PAE; families CE12 and CE13) remove acetyl groups from the O-2 and/or O-3 position of the α-(1,4)-linked d-GalA residues of the HG backbone (Benen et al., 2003) (Figure 1.4).

Figure 1.4. Schematic representation of the mode of action of pectin-degrading enzymes (adapted from Cankar et al. 2014).

Rhamnogalacturonan-degrading enzymes

Rhamnogalacturonan hydrolase (RGH; family GH28) hydrolyzes the α-1,2-linkage between GalA and rhamnosyl residues, releasing oligomers with Rha at their non-reducing ends (Mutter et al., 1994) (Figure 1.4). The presence of an acetyl substitution in the RG-I backbone hinders the action of RGH on RG-I (Kauppinen et al., 1995).

Rhamnogalacturonan lyase (RGL; families PL4 and PL11) mediates the beta-eliminative cleavage of the α-L-1,2-Rha*p*-α-D-1,4-Gal*p*A linkage within the RG-I backbone, forming 4,5-unsaturated bond at the non-reducing end (Mutter et al., 1998). Removal of acetyl groups also results in an increased RGL activity (Mutter et al., 1998). Rhamnogalacturonan acetylesterase (RGAE; family CE12) removes acetyl groups from the GalA residues in RG-I (Searle-van Leeuwen et al., 1992) and may also remove acetyl groups from HG (Bonnin et al., 2008).

Endo-galactanase (endoGAL; family GH53) cleaves the β-1,4-linkage between two unsubstituted galactosyl residues (Van de Vis et al., 1991) (Figure 1.4). β-Galactosidases (β-Gal; families GH2, GH35 and GH42) are exo-acting enzymes that degrade either β-(1,3)-, β-(1,4)-, or β-(1,6)-linked galactan, exclusively releasing single galactose residues from the non-reducing end of the molecules (Van de Vis, 1994) (Figure 1.4).

Endo-arabinanase (eARA; family GH43) is an endo-acting enzyme that hydrolyzes the linear α-1,5-linked arabinan (Figure 1.4) (Beldman et al., 1993; Kühnel et al., 2010). Exo-

arabinanases (families GH43 and GH93) release arabinose, arabinobiose, or arabinotriose from α-1,5-linked arabinan chains (Chávez Montes et al., 2008; Kühnel et al., 2010). Arabinofuranosidase (families GH43, GH51 and GH54) is able to release terminally linked arabinose moieties from the non-reducing end of arabinan or arabino-oligosaccharides (Inacio et al., 2008).

Xyloglucan-degrading enzymes

Xyloglucans can be degraded by xyloglucan-specific endo-glucanases (families GH5, GH12 and GH74), which cleave the linkages between unsubstituted Glc and xylose-substituted glucosyl residues (Pauly et al., 1999). The released oligomers always contain an unbranched Glc moiety at the reducing end (Hilz et al., 2007). Analysis of the released oligomers might provide valuable information regarding the different building blocks within the xyloglucan under investigation (Fry et al., 1993; Hilz et al., 2007).

Cellulose-degrading enzymes

The endo-(1,4)-β-D-glucanase (families GH5, GH9 and GH45) randomly attacks the internal O-glycosidic bonds, resulting in glucan chains of different lengths. The exo-(1,4)-β-D-glucanase (family GH55) acts on the ends of the cellulose chain and releases β-cellobiose as the end product. The β-glucosidases (family GH3) act specifically on the β-cellobiose disaccharides and produce Glc (Percival Zhang et al., 2006).

The architecture of plant cell walls

Since not much is known on potato cell wall architecture, the general view of plant cell walls is discussed to provide fundamental knowledge of potato cell wall architecture. The composition of cell wall varies during different plant developmental stages and between different parts of the plant (McCann et al., 1992). The cell wall polysaccharide network is a dynamic system that is modified continuously by plant endogenous enzymes to adapt to the environment or to provide the necessary structure during each plant development stage (Cosgrove, 2005). The primary cell wall is largely composed of cellulose, which is embedded in a matrix of pectin and hemicellulose (Keegstra, 2010). Pectin structural elements are believed to be covalently linked to each other (Vincken et al., 2003). Furthermore, pectin domains are also believed to be bind to xyloglucan by covalent and/or non-covalent bonds (Rizk et al., 2000; Cumming et al., 2005). In addition, for potato, RG-I galactan side chains have been observed to absorb to the surface of cellulose microfibrils, presumably mediated by hydrogen bonds (Zykwinska et al., 2005).

Cellulose microfibrils are directly linked to each another by xyloglucans that bind to the microfibril surface or perhaps become physically entrapped in the microfibril during its formation (Hayashi, 1989). Interaction of xyloglucan with pectin might occur during an

intermediate stage of cell wall component formation by plant biosynthesis (Tan et al., 2013). Xyloglucans are non-covalently linked with adjacent cellulose microfibrils to form a cellulose-xyloglucan network that constitutes the major load-bearing structure of the primary cell wall (Eckardt, 2008). These complex cell wall polysaccharide networks form the cell wall architecture. Additional studies are required to understand how polysaccharides are organized to form the complex architecture of plant cell walls.

Potato tuber texture

The texture of cooked potato tubers determines the consumer preference. Potato texture has been proposed to depend on the original cell structure of potato tissue (Van Marle et al., 1992). The cells of mealy potatoes have a round shape and can be easily separated after cooking (Van Marle, 1997). The presence of solubilized pectic polysaccharides into the cooking medium is closely correlated with the decrease in cell firmness (Van Marle et al., 1994). The cell wall polysaccharides from mealy texture potato exhibited more side chain structures than non-mealy type potato (Van Marle et al., 1997). The cell wall composition are believed to strongly correlate with the texture of cooked potatoes (Van Marle et al., 1992; Taylor et al., 2007), but the correlation between cell wall architecture and potato texture remains unclear.

In addition to the cell wall structure, the activity of endogenous enzymes also influences the potato texture. Analysis of the textural properties of cooked potatoes revealed a positive correlation between PME activity and fracture resistance during cooking (Ross et al., 2011). Cultivars with high PME activity showed resistance to fracturing during the cooking process through the formation of calcium-sensitive pectin structures.

Genetic resources for potato species

Potatoes exhibit greater genetic diversity than other crops, which may be reflected in the ability of potatoes to grow in a remarkably diverse range of environments (Hawkes, 1990). The genetic diversity of tetraploid potato (*Solanum tuberosum* L.) is a valuable resource for further improvement of the nutritional content, processing quality, yield and disease resistance of this crop (Bradshaw, 2007).

The generation of potato transgenic lines has been proven to be a very useful approach for elucidating the various factors that affect cell wall structures (Oomen, 2003). Numerically unreduced gametes are common in hybrids of haploid and diploid species (Yerk & Peloquin, 1989). Many wild-type potatoes cannot be crossed with cultivated potato varieties, which are mainly tetraploids, pentaploids or hexaploids (Bradshaw, 2007). Based on these reasons, the genetic modification of specific potato cell wall polysaccharides can

be used to investigate the function of these polysaccharides within the architecture of potato cell walls.

Genetic modification to alter the yield of plant cell wall

The complex genetic diversity of the tetraploid potato presents a challenge for potato breeders because several alleles spread across the four chromosome sets might control a trait (Tiemens-Hulscher et al., 2013). Changing the regulation of an endogenous gene or introduction of a heterologous gene can specifically modify the cell wall polysaccharides (Bradshaw, 2007). An approach to address the above challenges is the genetic modification via the expression of proteins responsible for *in vivo* modification of cell wall polysaccharides (Sørensen et al., 2000). Such modification can be achieved by either interfering directly with the enzymes involved in biosynthesis or by the modification of specific polymers through the expression of genes encoding for enzymes targeting these polymers (Martín et al., 2005; Øbro et al., 2009). There are multiple processes involved in the plant cell wall development and maintenance. Until now, the most common model plant used to study cell wall polysaccharide structure and modification is *Arabidopsis thaliana*, due to its relative small genome when compared to other plants (Caffall & Mohnen, 2009; Atmodjo et al., 2013). The candidate genes can be applied for modifying other plants.

Modification of potato cell wall polysaccharides

Several transgenic lines with modified potato pectin compositions have been reported and are summarized in Table 1.1. These transgenic lines have been generated via heterologous expression of various enzymes from fungi or plants that modify potato cell wall polysaccharides. Transgenic modifications of the potato cell wall polysaccharides have been targeted the pectin backbone, pectin side chains, pectin esterification and cellulose levels (Table 1.1).

Pectin backbone modification

Expression of RGL enzyme from *Aspergillus aculeatus* in the potato genome *in muro* reduces the amount of Gal and Ara in CWM (Oomen et al., 2002) (Table 1.1). The RGL transgenic lines have an abnormal development of the periderm of the elongated tubers (Oomen et al., 2002). The insufficient destarching procedure used in that study hampered the evaluation of cell wall polysaccharides.

Pectin side chain modificaton

Expression of endo-1,4-β-D-galactanase (endoGAL) from *Aspergillus aculeatus* (Sørensen et al., 2000) and β-Gal from *Cicer arietinum* (Martín et al., 2005) was meant to reduce the amount of Gal within potato tuber cell wall (Table 1.1).

The endoGAL transgenic lines had a lower Gal level (mol%), which was associated with less Gal in the side chains (Sørensen et al., 2000) (Table 1.1). The activity of β-Gal enzymes in the β-Gal transgenic lines leads to the reduction of Gal content in the cell walls (Martín et al., 2005). The reduction of Gal from cell walls corresponded to a reduction in 1,4-β-galactan side chains as measured by microscopy after immunolabeling of the galactans (Martín et al., 2005) (Table 1.1). Both endoGAL and β-Gal transgenic lines showed similar phenotype with that of the wild-type (Sørensen et al., 2000; Martín et al., 2005), indicating that the cell wall of potato tubers can tolerate reduced levels of galactan side chains.

The Ara presented in potato cell walls (mol%) could be reduced by 70% by expressing eARA from *Aspergillus aculeatus* (Skjøt et al., 2002) (Table 1.1). The low Ara level in cell walls correlated with less pectic arabinan side chains. The eARA transgenic lines did not show any visible altered phenotype in the potato tubers (Skjøt et al., 2002). The lacking information on the yield of CWM limited the understanding of the transgenic effects on whole potato transgenic line. The expression of genes encoding endoGAL or eARA enzymes lead to a reduced amounts of Gal or Ara (Ulvskov et al., 2005) (Table 1.1), which was associated with shorter galactan or arabinan side chains. The modified side chain structures caused the potato tubers to become more firm (Ulvskov et al., 2005). Making a double construct transgenic line (endoGAL + eARA) results in a simultaneous decrease in the amounts of Ara and Gal in *Arabidopsis thaliana* (Øbro et al., 2009) (Table 1.1). The same has been reported for potato transgenic lines (Cankar et al., 2014) (Table 1.1).

The uridine diphosphate glucose (UDP-Glc) 4-epimerase (UGE) enzyme catalyzes the reversible conversion of UDP-Glc and UDP-Gal (Holden et al., 2003), which are used as building blocks for the biosynthesis of galactan side chains (Maitra & Ankel, 1971). The UDP sugars can be directly or indirectly converted to other UDP sugar forms via the interconversion pathway (Reiter & Vanzin, 2001; Caffall & Mohnen, 2009; Bar-Peled & O'Neill, 2011). UGE transgenic lines showed an significant 2-3 fold increase of Gal in the potato tuber CWM (Oomen et al., 2004a) (Table 1.1).

Table 1.1. Origin of enzymes and their targeted polymers used in potato transgenic lines as well as reported effects on potato cell wall polysaccharides

Transgene-encoding enzymes	Source type	Species	Targeted polymers	Targeted effect on cell wall polysaccharides	Observations	References
Rhamnogalacturon lyase (RGL)	Fungal	*Aspergillus aculeatus*	Pectin backbone	Cleavage of RG-I backbone	Less galactan and arabinan[a]	Oomen et al. (2002)
β-Galactosidase (β-Gal)	Plant	*Cicer arietinum*	Pectin side chains	Shortening of galactan side chains of RG-I	Less galactan in cell wall[a]	Martin et al. (2005)
Endo-β-1,4-D-galactanase (endoGAL)	Fungal	*Aspergillus aculeatus*	Pectin side chains	Shortening of galactan side chains of RG-I	Less galactan in cell wall[a]	Sørensen et al. (2000)
Endo-α-1,5-L-arabinase (eARA)	Fungal	*Aspergillus aculeatus*	Pectin side chains	Shortening of arabinan side chains of RG-I	Less arabinan in cell wall[a]	Skjøt et al. (2002) Borkhardt et al. (2005)
Double construct (endoGAL + eARA)	Fungal	*Aspergillus aculeatus*	Pectin side chains	Shortening of arabinan and galactan side chains of RG-I	Not measured	Øbro et al. (2009) Cankar et al. (2014)
UDP-Glc 4-epimerase (UGE)	Plant	*Arabidopsis thaliana*	Pectin side chains	Elongation of galactan side chains of RG-I	Not measured	Oomen et al. (2004a)
Pectin methylesterase (PME)	Plant	*Petunia inflata*	Pectin esterification	Removal methyl esters of HG	Not measured (no effect methyl esters)	Pilling et al. (2000)
Pectin acetylesterase (PAE)	Plant	*Vigna radiata*	Pectin esterification	Removal acetyl groups of HG	Not measured (lower acetyl groups)	Orfila et al. (2012)
Sense cellulose synthase (CesA)	Plant/ bacterial	*Gossypium hirsutum, Oryza sativa, Acetobacter xylinum*	Cellulose	Increase in cellulose level	Cellulose increase[b]	Oomen et al. (2004b)
Anti-sense cellulose synthase (asCesA)	Plant/ bacterial	*Gossypium hirsutum, Oryza sativa, Acetobacter xylinum*	Cellulose	Decrease in cellulose level	Cellulose increase[b]	Oomen et al. (2004b)
Class-specific regions of cellulose synthase (CSR)	cDNA of CesA		Cellulose	Down regulation of the RNA expression levels of CesA gene to decrease cellulose level	Cellulose decrease[b]	Oomen et al. (2004b)

[a]The level of side chain were measured using microscopy after antibody immunolabeling.
[b]The level of cellulose was measured by colorimetrical assay.

Pectin ester modification

The acetylation of cell wall pectin in PAE transgenic line was reduced by 68% after the introduction of mung bean PAE into potato tubers (Orfila et al., 2012). The de-acetylation of cell wall pectin had no effect on the phenotype, but the tuber tissue became stiffer (Orfila et al., 2012). The manipulation of plant PME expression into potato genome did not significantly change the methyl-esterification of cell wall pectin (<5%) (Pilling et al., 2000). However, even without any effect on the methyl-esterification, the stems of PME transgenic lines elongated more rapidly and showed a lower tuber yield than their wild-type counterparts (Pilling et al., 2000).

Cellulose modification

Oomen et al. (2004b) used CesA cDNA for producing CesA and antisense constructs (asCesA) to respectively increase or decrease cellulose levels in potato (Table 1.1). Unlike many other plants and plant systems, potato tubers did not show any phenotypic alterations after the decrease in cellulose level (Oomen et al., 2004b). Oomen et al. (2004b) used the class-specific region (CSR) to specifically downregulate the corresponding CesA genes, which resulted in the reduction of approximately 50% of cellulose content compared to that of the wild-type (Table 1.1)

Polysaccharides structural information is limited for transgenic potato

Alterations of potato cell walls based on the pectin backbone, pectin side chains, pectin esterification and cellulose levels of CWM from wild-type potato varieties and their transgenic lines have already been investigated. Difficulties associated with the CWM isolation steps have hampered the detailed studies of the modified polysaccharides of cell walls (Jarvis et al., 1981; Van Marle et al., 1997; Sørensen et al., 2000; Jardine et al., 2002; Oomen et al., 2004a). Moreover, the unsuccessful starch removal from CWM also hampered the further fractionation and characterization of potato CWM.

The incomplete information in the literature on cell wall polysaccharide of transgenetically modified potatoes, such as CWM yield, fresh weight-based monosaccharide levels, or molar monosaccharide compositions, has limited the evaluation of effects of such transgenic modifications on potato tuber cell walls. Modified potato cell wall polysaccharides have only been qualitatively analyzed using microscopic techniques in combination with antibody immunolabeling (Knox et al., 1990; Jones et al., 1997; Willats et al., 1998) and Fourier-transform infrared microspectroscopy (Chen et al., 1998). Other evaluation techniques have also been used. However, each technique provides its own type of useful information on the modifications resulting from genetic engineering, which could not always be compared with each other easily.

The molar monosaccharide compositions of cell wall polysaccharides are often used in combination with qualitative techniques, although the lack of information on CWM hinders the detailed elucidation of the effect on potato tuber after transgenic modification. Furthermore, the acetylation and methyl-esterification of modified cell wall pectin have been often ignored when these characteristics were not the target. Therefore, a strategy for the more complete evaluation of wild-type potato and its transgenic lines is necessary to overcome these problems.

Thesis outline

The main aim of this study is to elucidate the polysaccharide structures of cell walls of wild-type potato varieties and their transgenic lines. The current procedure for the isolation of potato CWM needs to be improved, especially with respect to the starch-removal steps. Separation procedures following CWM isolation are introduced to obtain various individual polysaccharide populations. Characterization of the extracted polysaccharides will enable the evaluation of transgenic modification on potato cell wall polysaccharides.

Previous studies have provided data regarding the monosaccharide composition of potato CWMs of both β-galactosidase (β-Gal-14, targeting pectin side chains) or rhamnogalacturonanlyase (RGL-18, targeting pectin backbone) transgenic lines, respectively. But, this information was insufficient to fully describe the effect of transgenic modification. In this study, CWM was extracted from Karnico (wild-type) and its β-Gal-14 and RGL-18 transgenic lines. Pectic polysaccharides from CWMs of these potato varieties were isolated and characterized **(Chapter 2)**. Two populations of xyloglucan fractions were extracted from the CWMs of Karnico and two of its transgenic lines (**Chapter 3**). The characterization of the xyloglucan structures can provide information of the effect of pectin transgenic modification on other cell wall polysaccharides. Subsequently, UGE transgenic lines with Kardal (wild-type) background having a modified pectin side chain synthesis were characterized by their cell wall polysaccharides (**Chapter 4**).

Based on the findings of the in-depth analysis performed (**Chapters 2, 3 and 4**), a strategy was developed to introduce structural parameters for screening potato transgenic lines (**Chapter 5**). The method was initially used to evaluate potato transgenic lines based on the following categories of cell wall transgenic modification: (A) pectin side chains, (B) pectin backbone, (C) pectin esterification and (D) cellulose level. The transgenic-targeted structures and other polysaccharides were analyzed for an overall understanding of the transgenic modification of potato. Lastly, the influence of the modified cell wall structure on potato texture and physical properties is described (**Chapter 6**). The impact of transgenic modification on potato CWMs and research needed for cell wall structural identification are discussed.

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Chapter 2

Modification of potato cell wall pectin by the introduction of rhamnogalacturonan lyase and β-galactosidase transgenes and their side effects

Abstract

Genes encoding pectic enzymes were introduced to wild-type potato Karnico. Cell wall materials were extracted from Karnico and transgenic lines expressing β-galactosidase (β-Gal-14 transgenic line) or rhamnogalacturonan lyase (RGL-18 transgenic line). After sequential extraction, β-Gal-14 hot buffer-soluble solids (HBSS) of pectin contained 54% less galactose than Karnico HBSS, representing shorter galactan side chains. The individual pectin populations of β-Gal-14 HBSS showed different modifications extended to the two sub-populations as obtained by ion-exchange chromatography. Compared to wild-type, RGL-18 HBSS contained 27% more galacturonic acid and 55% less Gal on fresh potato weight basis, which was due to the removal of galactan-rich rhamnogalacturonan I (RG-I) segments. All pectin populations of RGL-18 showed consistently low levels of RG-I segments. Transgenic modification showed side effects on the methyl-esterification and acetyl substitution of RGL-18 HBSS (DM = 53, DA = 21), but not of the β-Gal-14 HBSS in comparison to wild-type (DM = 29, DA = 54).

Introduction

Pectin is a major component of primary plant cell walls and is primarily composed of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). The chemical structure of pectic polymers influences plant growth and development (Ridley et al., 2001). HG is a linear polymer composed of α-(1→4)-linked galacturonosyl residues, whereas the RG-I backbone is composed of repeating α-(1→2)-L-rhamnose (Rha)-α-(1→4)-D-galacturonic acid (GalA) units. Neutral side chains composed predominantly of (1→4)-linked β-D-galactose (Gal) and/or (1→5)-linked L-arabinose (Ara) residues are attached at the O-4 position of Rha within the RG-I backbone (Schols & Voragen, 1994). The GalA residues of potato HG are reported to be methyl esterified at C-6 for around 40%, while no indications are present that GalA residues in the RG-I segment could be methyl esterified (Ross et al., 2011). In contrast to most pectins from other sources, potato pectin is acetylated within the HG segments, especially at the *O*-3 position of the GalA residues (Ishii, 1997). In addition, acetyl groups were also found to be present in potato RG-I, attached predominately to *O*-2 of the GalA residues (Ishii, 1997). Furthermore, potato pectin can be characterised by long β-1-4-galactan rich side chains of RG-I (Sørensen et al., 2000), although Øbro et al. (2004) reported that arabinan side chains, branched with arabinose and even galactose could be present.

Modifications in the final structure of potato pectin can alter its characteristics, changing the quality of potato product (Ross et al., 2011). In order to understand cell wall polysaccharide structure, architecture and biosynthesis, one approach used in potato research has been to transgenetically change the polysaccharides and to examine changes observed in the cell wall material. Introducing a β-galactosidase (β-Gal) encoding gene from *Cicer arietinum* into the potato genome results in removal of the galactan side chains from RG-I (Martín et al., 2005). Expression of a rhamnogalacturonan lyase (RGL) encoding gene from *Aspergillus aculeatus* causes the RGL to act on the backbone of rhamnogalacturonan I (RG-I). The RGL transgenic lines have markedly lower Gal and Ara contents and show an abnormal periderm development (Oomen et al., 2002).

Previous research has provided data regarding the monosaccharide composition of potato tuber cell wall material (CWM) of both β-Gal and RGL transgenic lines. For both lines the enzyme expression resulted in lower Gal levels in the cell wall polysaccharides of the potato tubers (Oomen et al., 2002; Martín et al., 2005). This analysis could not be completed, due to difficulties with complete removal of starch from CWM, which hampered detailed studies of the modified cell wall polysaccharides (Oomen et al., 2002). To understand the effects of lower Gal levels in pectin composition and properties, β-Gal (side chain modification) and RGL (pectin backbone modification) were overexpressed in potato cv. Karnico.

In this manuscript we describe a detailed analysis of cell wall polysaccharides in the transgenic plants. After having optimized the destarching process during CWM isolation, the constituent monosaccharide composition, side chain length, molecular weight (M_w) distribution and esterification levels of individual pectin populations of CWM from both transgenic lines were compared to that of Karnico. The results provide a clear view of the effect of two transgenic lines on their individual pectin populations.

Experimental procedures

Materials

Potato transgenic lines

The cDNA vector pYES2/eRGL, containing either the gene encoding RGL from *Aspergillus aculeatus* (Kofod et al., 1994) or the gene encoding β-Gal from *Cicer arietinum* (Martín et al., 2005), was used to transform potato cv. Karnico and generate the transgenic lines RGL-18 and β-Gal-14, respectively. Karnico plants transformed with the empty vector or untransformed were used as control. Potato tubers were grown in a greenhouse (Unifarm, Wageningen UR, Wageningen, The Netherlands) and harvested in December 2011. Potato tubers were peeled and diced into 5-mm cubes, then rapidly frozen in liquid nitrogen to preserve enzyme activity. The samples were stored at -20°C until use.

Isolation of cell wall materials

Frozen potato cubes (250 g) were mixed with 500 mL sodium acetate buffer (0.2 M, pH 5.2) and immediately homogenised with a hand-held disperser. The resulting slurry was heated to 80°C and held for 30 min to promote starch gelatinisation and to inactivate endogenous potato enzymes. The samples were then incubated at 40°C for 3 h with 2000 U α-amylase from *Bacillus licheniformis* (Sigma-Aldrich, St. Louis, MO, USA) and 2000 U amyloglucosidase from *Rhizopus* mould (Sigma-Aldrich). Subsequently, cell wall polysaccharides were precipitated by adding ethanol to a final concentration of 70% (v/v). After centrifugation (15 min; 10,000 × g; 4°C), the pellet obtained was resuspended in buffer and subjected to the heating and enzymatic degradation procedures another two times. The final pellet was washed with 70% ethanol until no sugars were present in the supernatant. The washed pellet was lyophilised, yielding CWM.

Sequential extraction of CWM

The potato CWM was sequentially extracted: hot buffer-soluble solids (HBSS) were extracted using 0.05 M sodium acetate buffer (pH 5.2) at 70°C, chelating agent-soluble solids (ChSS) were extracted using 0.05 M EDTA and 0.05 M sodium oxalate present in 0.05 M sodium acetate buffer (pH 5.2) at 70°C, and diluted alkali-soluble solids (DASS) were extracted using 0.05 M sodium hydroxide with 0.02 M sodium borohydride at 0°C.

The exact conditions are described elsewhere (Vierhuis et al., 2000). Fractions containing ChSS were first dialysed against ammonium acetate buffer (0.1 M, pH 5.2) for 18 hours then dialysed again with distilled water and freeze-dried. Other extracts were obtained after dialysis against distilled water directly and freeze-dried. The final residues (RES) were washed with distilled water and freeze-dried.

Analytical methods

Constituent monosaccharide composition

A pre-hydrolysis step was carried out using 72% (w/w) sulphuric acid at 30°C for 1 h. The samples were subsequently hydrolysed with 1 M sulphuric acid at 100°C for 3 h and sugars released were analysed as their volatile alditol acetates by GLC as described previously (Englyst & Cummings, 1984). The total uronic acid content was determined using an automated-m-hydroxydiphenyl assay (Thibault, 1979). The total uronic acid content measured was attributed to GalA.

Starch content of CWM was analysed enzymatically using the Total Starch Kit from Megazyme (Wicklow, Ireland). The starch glucose (Glc) content of CWM was subtracted from the total Glc content of the CWM to obtain the cell wall Glc content.

The molar constituent monosaccharide composition of each fraction was used to calculate various polymer characteristics. The molar ratio of Gal:Rha represents the average length of the galactan side chain. The pectin backbone is composed of HG (100% GalA) and RG-I (Rha:GalA ratio of 1:1); the relative proportions of these structural backbone elements can be inferred from the molar ratios of Rha and GalA by: HG = GalA – Rha and RG-I = 2 × Rha.

Degree of acetylation and methyl esterification

Acetyl groups present on the polysaccharides were determined using high-performance liquid chromatography (HPLC) after saponification in sodium hydroxide (Voragen et al., 1986). Pectin samples were saponified in sodium hydroxide to determine the degree of methyl esterification (DM) using a colorimetric method, as previously described (Guillotin et al., 2007). The degrees of acetylation (DA) and DM were calculated as the moles of acetyl groups and methyl esters per 100 moles of GalA, respectively.

Molecular mass distribution

The molecular mass distribution of extracted pectin fractions was analysed using high performance size exclusion chromatography (HPSEC) on an Ultimate 3000 HPLC system coupled to a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The HPSEC columns were the TSK gel Super AW4000, AW3000 and AW2500 columns in series, and the guard column was a Super AW-L (Tosoh Bioscience, Tokyo, Japan). The columns were

kept at 55°C and 0.2 M $NaNO_3$ was used as eluent with flow rate of 0.6 mL/min. Pullulan standards (Sigma-Aldrich) were used for estimating the molecular mass of the compounds eluted.

Degradation levels of high molecular weight pectin population were calculated in the range of 7 -10.5 min (correlated to $\pm 3 \times 10^6$ till $\pm 4 \times 10^4$ Da) for Karnico and β-Gal-14 and 7 -12.5 min ($\pm 3 \times 10^6$ till $\pm 3 \times 10^3$ Da) for RGL-18. The level of degradation for Karnico and β-Gal-14 was defined as area of 7-10.5 min after enzymatic degradation / area of 7-10.5 min without enzymatic degradation. For RGL-18, the degradation was defined as area of 7-12.5 min after enzymatic degradation / area of 7-12.5 min without enzymatic degradation.

Enzymatic degradation of HBSS fractions

The HBSS fractions were dissolved (5 mg/mL) in 50 mM sodium acetate buffer (pH 5.2). Subsequently, the fractions were digested with endopolygalacturonase (EC 3.2.1.15) from *Aspergillus aculeatus* (PG) (Limberg et al., 2000), pectin methylesterase (EC 3.1.1.11) from *Aspergillus niger* (PME) (Van Alebeek et al., 2003) and endo-β-1,4-galactanase (EC 3.2.1.89) from *Aspergillus aculeatus* (endoGAL) (Van de Vis et al., 1991). Sufficient enzyme was added to degrade the corresponding substrate in 6 hours at 40°C, and overnight incubation was done to ensure complete degradation.

Anion exchange chromatography for separation of pectins

Bound and non-bound pectins present in HBSS fractions of wild-type (Karnico) and transgenic (RGL-18 and β-Gal-14) potatoes were separated using anion exchange chromatography (AEC). Samples of HBSS fractions (15 mg) were dissolved in 3.5 mL Milli-Q water. The AEC column was packed using Dowex 1X8 (Fluka, St. Louis, MO, USA) in the acetate (OAc^-) form into a 5 mL pipet tip with cotton as the frit material. The sample solution (3 mL) was loaded onto the AEC column and eluted under gravity. Subsequently, 3 mL Milli-Q water was added twice to elute the non-bound pectin remaining on the column and collected separately. Subsequently, 3 mL of 0.5 M sodium acetate buffer (pH 5.0) was added three times to elute bound pectin and collected separately.

The fractions were analysed for constituent monosaccharide composition separately. The data from bound or non-bound pectin fractions were combined into B-fraction or NB-fraction, respectively.

Results and Discussion

CWM composition in transgenic lines relatively to the Karnico control

In previous studies, the residual starch content in isolated CWM limited the study of cell walls (Sørensen et al., 2000; Oomen et al., 2002; Orfila et al., 2012). The starch contents of Karnico, β-Gal-14 and RGL-18 tubers were 78.2%, 82.4% and 80.1% w/w on dry matter basis, respectively. The starch was removed by more than 99.5%, resulting in residual starch levels in the CWM of 4.1% in β-Gal-14 and RGL-18, and 5.8% in Karnico. This allowed us to calculate cell wall Glc levels and to characterize various pectin populations accurately.

To elucidate the effects of transgenic modifications on potato cell wall, the CWMs from wild-type and transgenic lines were analysed for their constituent monosaccharide compositions (Table 2.1). Polysaccharides present in all three CWMs consisted primarily of Gal, Glc and GalA. To understand the effects of transgenic modifications on potato cell walls, we not only compared the monosaccharide composition, but also calculated the yield per individual sugar present in the total CWM fraction on a fresh potato tuber basis (mg per 100 g tuber, Table 2.2).

Karnico (wild-type) versus β-Gal-14: The cell wall carbohydrates content in the CWM preparations of β-Gal-14 (1389.9 mg/100 g tuber) was 41% lower than in Karnico (2347.2 mg/100 g tuber). The molar proportions of Gal in the Karnico and β-Gal-14 CWMs were quite similar: 25.8 and 23.1 mol %, respectively (Table 2.1). Nevertheless, they represented different absolute amounts when expressed in terms of fresh tuber weight (Table 2.2): 605.1 and 319.8 mg/100 g tuber for Karnico and β-Gal-14, respectively. This corresponds with a 47% decrease upon introduction of the β-Gal gene. A decrease in Gal content due to introduction of a β-Gal gene has been previously reported, albeit only qualitatively (Martín et al., 2005). The Glc and GalA content in β-Gal-14 were reduced by 43% and 23%, respectively, compared to wild-type (Table2). However, the *molar* GalA content showed an increase from 12.6 mol % in Karnico to 16.4 mol % in β-Gal-14 (Table 2.1). Martín et al. (2005) indicated that the level of transgenic β-Gal expression has no effect on the amount of cellulose. The Glc and carbohydrate contents decreased 43% and 41%, respectively, in β-Gal-14 compared to Karnico. The decrease of Glc might be caused by a lower amount of CWM in β-Gal-14 during tuber growth. All sugars decreased by ±40% in β-Gal-14 CWM except GalA. Based on the constituent monosaccharide composition, the β-Gal transgenic line had a reduction of the targeted monosaccharides (Gal), while the other monosaccharides changed in proportion.

Karnico (wild-type) versus RGL-18: The carbohydrate content in fresh tubers showed a 35% reduction in RGL-18 CWM compared to Karnico CWM (Table 2.2). The reduced Rha

Table 2.1. Molar monosaccharide composition (mol %) and degree of acetylation (DA) and methyl esterification (DM) of cell wall material and its fractions of Karnico (wild-type) and transgenic β-Gal-14 and RGL-18 potato tubers.

Name	mol %							DM (%)[a]	DA (%)[a]	Carbohydrate content (% w/w)
	Rha	Ara	Xyl	Man	Gal	Glc	GalA			
Karnico-CWM	0.7	3.1	1.7	1.0	25.8	55.1[b]	12.6	41	29	66.1
Karnico-HBSS	1.2	6.7	0.5	0.6	70.4	3.2	17.4	29	54	71.8
Karnico-ChSS	1.1	5.4	1.0	1.1	36.7	12.6	42.1	19	16	56.9
Karnico-DASS	0.9	4.4	0.9	3.0	28.9	47.2	14.7	-[c]	-	22.6
Karnico-RES	0.5	2.3	2.9	1.7	10.8	79.8	2.0	-	-	70.6
β-Gal-14-CWM	0.9	3.5	2.3	1.2	23.1	52.6[b]	16.4	36	20	52.1
β-Gal-HBSS	1.9	6.4	1.3	1.1	50.7	16.4	22.2	30	51	60.5
β-Gal-ChSS	1.2	6.6	1.7	1.7	22.3	24.2	42.3	25	23	35.3
β-Gal-DASS	0.8	5.8	2.0	4.4	33.8	40.8	12.4	-	-	12.7
β-Gal-RES	0.6	2.4	3.5	1.7	9.5	80.3	2.0	-	-	65.4
RGL-18-CWM	0.6	3.9	2.6	1.9	13.6	55.3[b]	22.1	43	16	57.9
RGL-HBSS	1.6	5.1	0.8	1.2	51.3	4.4	35.6	53	21	69.8
RGL-ChSS	1.0	7.3	1.1	1.4	8.8	10.2	70.2	17	7	47.0
RGL-DASS	0.8	5.2	1.4	3.0	7.8	54.0	27.8	-	-	19.9
RGL-RES	0.4	3.8	4.5	3.1	3.7	81.8	2.7	-	-	58.4

Abbreviations: CWM = cell wall materials; HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids; RES = extraction residues.
[a]Number of moles of acetyl or methyl per 100 moles of galacturonic acid.
[b]Glc content after starch correction. [c]Not determined.

Table 2.2. Yield, constituent cell wall monosaccharide in cell wall materials (CWM) and its fractions from Karnico (wild-type), β-Gal-14 and RGL-18 potatoes based on fresh potato tuber basis.

Name	Yield (g/100 g CWM)	mg/ 100g tuber							
		Rha	Ara	Xyl	Man	Gal	Glc	GalA	Total carbohydrate
Karnico-CWM		15.3	59.3	32.6	23.2	605.1	1291.2[a]	320.5	2347.2
Karnico-HBSS	19.4	5.2	26.4	1.9	2.9	343.6	15.8	91.9	487.7
Karnico-ChSS	2.8	0.6	2.4	0.4	0.6	19.9	6.8	24.9	55.6
Karnico-DASS	12.2	0.8	3.5	0.7	2.9	28.3	46.2	15.6	98.1
Karnico-RES	39.2	4.7	18.8	23.4	16.8	107.0	789.9	21.3	982.0
β-Gal-14-CWM		10.9	39.5	25.6	17.0	319.8	729.8[a]	247.3	1389.9
β-Gal-HBSS	22.8	5.4	16.3	3.3	3.5	158.1	51.2	75.2	312.9
β-Gal-ChSS	1.6	0.3	1.4	0.3	0.4	5.8	6.3	11.9	26.4
β-Gal-DASS	15.0	0.3	2.0	0.7	1.8	14.1	17.0	5.6	41.3
β-Gal-RES	37.2	3.4	13.3	19.5	12.0	65.6	554.8	15.2	683.9
RGL-18-CWM		8.8	47.8	32.3	28.7	206.5	840.4[a]	364.9	1529.4
RGL-HBSS	16.2	4.3	12.6	2.1	3.7	154.2	13.2	116.4	306.4
RGL-ChSS	2.4	0.3	2.0	0.3	0.5	2.9	3.4	25.4	34.8
RGL-DASS	11.4	0.5	2.7	0.7	1.9	5.0	34.2	19.2	64.1
RGL-RES	45.7	2.2	19.0	22.7	18.8	22.7	501.2	17.9	604.4

Abbreviations: HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids; RES = extraction residues.
[a]Glc content after starch correction. The starch content: Karnico-CWM: 5.8%, β-Gal-14-CWM: 4.1% and RGL-18-CWM: 4.1%.

level (42% decrease) in the RGL-18 CWM compared to wild-type, corresponds to a reduction of the RG-I structural element level of the pectin backbone. In contrast, the GalA content of the RGL-18 was 14% higher than that of Karnico. These combined effects indicate that the pectin structure has been altered for RGL-18 towards a higher HG:RG-I ratio. Oomen et al. (2002) indicated similar effects on the Rha and GalA levels (mol%), but information was lacking on the total amount of polysaccharides present in the tubers. Hence, no comparison based on fresh tuber weight of wild type and transgenic lines was given. In our study, the RGL-18 showed 66% less Gal than Karnico (Table 2.1). The RGL cleaves the RG-I backbone specifically (Mutter et al., 1998), removing part of RG-I backbone, which may still have long galactan side chains attached.

Esterification of CWM from wild-type and transgenic lines

Methyl-esterification of Karnico pectin (DM = 43; Table 2.1) was similar as for RGL (DM = 41), but slightly lower for β-Gal-14 (DM = 36). Acetylation was lower in β-Gal-14 (DA = 20) and RGL-18 (DA = 16) than in Karnico (DA = 29). Pectin esterase and pectin estertransferase activities might have changed after β-Gal or RGL transgenic modification resulting in the modified DM and DA levels of pectin from the transgenic lines. However, precise mechanisms are not known.

Sequential extraction of potato CWM

The constituent monosaccharide composition of potato CWM alone does not provide detailed information on the structure of individual pectin populations. Therefore, sequential extraction of the CWM was performed to isolate different pectin populations (HBSS, ChSS and DASS) and RES. The HBSS fraction was the most abundant pectin fraction of all three potato varieties (Table 2.2). The recovery on weight base of the sequential extraction was 69%, 77% and 66% for Karnico, β-Gal-14 and RGL-18, respectively. Despite losses during the extraction procedures, the fractions obtained are considered to sufficiently represent the CWM and fractions will be used for detailed characterization as reported before (Sengkhamparn et al., 2009). The same evaluation procedure as for CWM from wild-type and transgenic lines was carried out for each pectin fraction.

Buffer soluble pectin

The Gal:Rha ratios of HBSS fractions were 58.7:1 in Karnico vs. 26.7:1 in β-Gal-14 (Table 2.3). The effect of the β-Gal on shortening the side chain is much more visible in the HBSS fraction than in the CWM, where it was 36.9:1 in Karnico and 25.7:1 in β-Gal-14 (Table 2.3). The Gal:Rha ratio of Karnico CWM was similar as found for other wild-type potato tubers (Van Marle et al., 1997). According to the HG:RG-I ratio, the β-Gal transgenic line not only showed shorter galactan side chains, but also exhibited a modified pectin backbone structure of the HBSS fraction. The β-Gal HBSS had the same level of DA

and DM with Karnico HBSS (Table 2.1). The unexpected effects on non-targeted monosaccharide and pectin backbone have to be considered after transgenic modification as the side effects of β-Gal transgenic line.

RGL-18 HBSS showed a 17% decrease of its Rha content compared to Karnico HBSS (Table 2.2), indicating a reduction in RG-I regions by the action of the RGL enzyme. The low Gal:Rha ratio (Table 2.3) may have been caused by cleavage of the galactan-rich RG-I backbone rather than cleavage of the galactan chains. Release of the RG-I backbone segments in combination with an increase in HG regions (in addition to a 27% GalA content, Table 2.2) resulted in a quite different pectin in RGL-18 HBSS than in Karnico HBSS. The RGL-18 HBSS showed completely different esterification levels (DM = 53%, DA = 21%) than Karnico HBSS. RGL-18 HBSS has apparent effects on DM and DA than in the CWM. The side effects on pectin esterification after introducing the RGL gene are unclear.

Table 2.3. Galactose (Gal):rhamnose (Rha) ratios and Homogalacturonan (HG):Rhamnogalacturonan I (RG-I) ratios of Karnico (wild-type) and transgenic β-Gal-14 and RGL-18 potatoes in as well as polysaccharide extracts and residues of each variety.

	Karnico		β-Gal-14		RGL-18	
	Gal:Rha	HG:RG-I	Gal:Rha	HG:RG-I	Gal:Rha	HG:RG-I
CWM	36.9	8.5	25.7	8.6	22.7	17.9
HBSS	58.7	6.8	26.7	5.3	32.1	10.6
ChSS	33.4	18.6	18.6	17.1	8.8	34.6
DASS	32.1	7.7	42.3	7.3	9.8	16.9
Sub-populations of HBSS						
Non-bound pectin (NB-fraction)	30.0	1.3	13.3	0.9	15.4	3.1
Bound pectin (B-fraction)[a]	4.6	4.2	3.4	4.2	1.5	5.5

Abbreviations: HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids.

Calcium bound pectin

High HG:RG-I ratios in the ChSS fractions illustrated the presence of HG rich pectin in all three potato samples (Table 2.3). A shorter side chain length (lower Gal:Rha ratio) was seen for β-Gal-14 ChSS (18.6:1 vs. 33.4:1 in Karnico ChSS), while the HG:RG-I ratio stayed the same. For RGL-18 ChSS, a low Gal:Rha ratio in combination with a lower proportion of RG-I (higher HG:RG-I ratio) indicates the release of galactan-rich RG-I regions upon introduction of the RGL gene (Table 2.3). The remaining RG-I backbone in RGL-18 ChSS might be considered to be highly acetylated, which could be explained by a low efficiency of RGL on releasing acetylated parts of RG-I (Mutter et al., 1998). The

higher DM and DA in β-Gal-14 ChSS compared to Karnico ChSS (Table 2.1), indicate different pectin properties for wild-type and transgenic line ChSS fractions.

DASS fractions

The DASS fractions yielded 11.4% - 15.0% from CWM (Table 2.2). The low carbohydrate content was found for all potato varieties (12.7% - 22.6%, Table 2.1). The Glc content in DASS fractions also included residual starch (not determined), which was present in small quantities in the CWM and was solubilised under the alkaline conditions.

The diluted alkali solution may have released some galactan-rich pectins, which were linked to the (hemi)cellulose network though esters and hydrogen bonds (Zykwinska et al., 2005). In contrast to CWM, HBSS or ChSS, the side chain length in the DASS fraction from β-Gal-14 (Gal:Rha ratio of 42.3) was found to be longer than that from Karnico (Gal:Rha ratio 32.1) (Table 2.3). The β-Gal enzyme clearly modified the various pectin populations differently.

The RGL-18 DASS showed a high HG:RG-I ratio and a low Gal:Rha ratio (Table 2.3). The combination indicated that galactan-rich RG-I backbones were released by the RGL enzyme. The RGL enzyme showed a consistent effect on pectin backbone of RGL transgenic line in the pectin populations HBSS, ChSS and DASS.

Extraction residues

Glc was the dominant monosaccharide in all three RES fractions (Table 2.2). The presence of Ara, xylose (Xyl), Gal and GalA is indicative for strong interactions between pectin and (hemi)cellulose (Zykwinska et al., 2005). Gal contents in the β-Gal-14 RES and RGL-18 RES were 39% and 79% lower, respectively, than in Karnico RES (Table 2.2). The monosaccharide compositions for wild-type and β-Gal-14 RES are similar, but are quite different from RGL-18 RES (Table 2.1). RGL-18 RES differs in Ara (3.8 mol%), Xyl (4.5 mol%) and Gal (3.7 mol%) when compared to Karnico and β-Gal-14 RES (Table 2.1), which might indicate the differences of building blocks of xyloglucan. However, further xyloglucan purification steps must be performed to prove this hypothesis.

In depth characterisation of HBSS pectins

Since the HBSS fractions were extracted in much higher yields compared to the ChSS and DASS fractions (Table 2.2), these extracts were studied in more detail for their molecular weight distribution and their degradability by pure and well defined enzymes.

Enzymatic degradation of HBSS fractions

Size exclusion chromatography was used to analyse the Mw distribution of polysaccharides in the HBSS fractions before and after enzymatic degradation.

The Karnico HBSS exhibited a main population around 7×10^5 Da, while the Mw distribution of the β-Gal-14 HBSS exhibited two populations, around 7×10^5 Da and 3×10^5 Da (Figure 2.1A). Cleavage of the side chain by the β-Gal enzyme does not change the Mw distribution in the range of $\pm 1 \times 10^5$ Da to $\pm 3 \times 10^6$ Da. In contrast, the RGL-18 HBSS exhibited a considerably broader Mw distribution pattern with a main population at $\pm 6 \times 10^4$ Da (Figure 2.1A), which might be caused by partial hydrolysis of the RG-I backbone by the RGL enzyme.
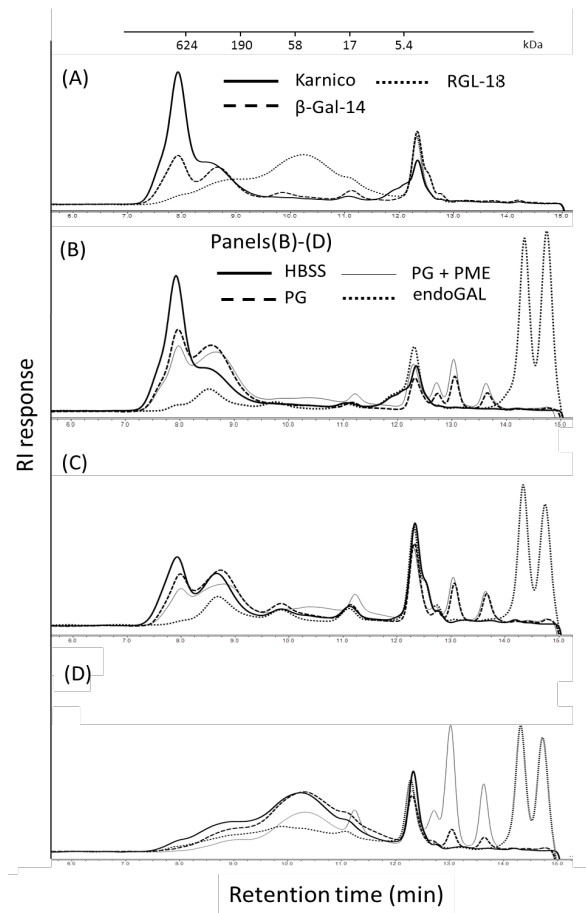
Figure 2.1. HPSEC profiles of (A) HBSS fractions from Karnico and β-Gal-14 and RGL-18 transgenes and HBSS fractions digested with endopolygalacturonase (PG), PG in combination with pectin methylesterase (PME) and β-endogalactanase (endoGAL) from (B) Karnico, (C) β-Gal-14 and (D) RGL-18. Molecular masses of pullulan standards (in kDa) are indicated.

To verify the chemical fine structure of both the HG segments and the galactan side chains, HBSS fractions from wild-type and transgenic line potatoes have been enzymatically degraded. As shown in Figure 2.1B, Karnico HBSS can be degraded by PG, PG + PME and endoGAL. Table 2.4 summarizes the digestion levels by each enzyme as calculated from the peak areas of the high M_w population before and after enzyme degradation. After PG degradation, the M_w of the pectin was only partly shifted (from $\pm 7 \times 10^5$ Da to $\pm 2 \times 10^5$ Da), and a minor amount of degradation products were eluted around $\pm 3 \times 10^3$ Da. The high M_w peak area of the Karnico HBSS PG digest showed a 6% reduction compared to the peak area of the parental Karnico HBSS (Table 2.4). The combination of PG and PME resulted in a peak area reduction of 22%. The methyl-esterified galacturonic acid residues of Karnico HBSS might be partly block-wise distributed over the HG chain. After PME hydrolysis, PG can approach GalA sequences more readily, which results in a better degradation than using PG alone.

Table 2.4. Degradation levels of high molecular weight pectin population by endopolygalacturonase (PG), PG combined with pectin methylesterase (PME) and β-endogalactanase (endoGAL) of hot buffer-soluble solids (HBSS) fractions from potatoes (Karnico, β-Gal-14 and RGL-18).

Sample	PG (%)[a]	PG + PME (%)[a]	endoGAL (%)[a]
Karnico-HBSS	6	22	81
β-Gal-14-HBSS	13	32	70
RGL-18-HBSS	12	51	62

[a]Values were calculated from the peak area ratios of high molecular weight HBSS fractions before and after enzymatic digestion within the time windows for 7-10.5 min (Karnico and β-Gal-14) and 7-12 min (RGL-18).

Gal is the most abundant monosaccharide present in Karnico HBSS (Table 2.1). The use of endoGAL (β-(1→4)-linked galactan-degrading enzyme) resulted in 81% degradation (Table 2.5). This result proved that the isolated potato pectin was rich in β-(1→4)-linked galactan, as described previously (Sørensen et al., 2000).

In addition, the M_w distribution of β-Gal-14 HBSS and RGL-18 HBSS and their digests are shown in Figures 2.1C and 2.1D, respectively. β-Gal-14 HBSS showed higher degradation levels of the high M_w population by PG or PG + PME than found for the wild-type (Table 2.4). This effect could have been caused by the higher GalA level present in β-Gal-14 HBSS (22.2 mol%) than in Karnico HBSS (17.4 mol%, Table 2.1). Karnico and β-Gal-14 HBSS showed the same levels of esterification and molar proportion of GalA (Table 2.1), but the distribution of methyl esterification might differ over the pectin backbone, resulting in more degradation for β-Gal-14 HBSS. The RGL-18 HBSS exhibited 12% degradation of the high M_w population following exposure to PG alone. A 51% degradation was found after treatment by PG + PME. The degradation level by PG + PME suggests that the pectin structure of RGL-18 HBSS is different from wild-type.

Moreover, endoGAL digestion of β-Gal-14 and RGL-18 HBSS resulted only in 70% and 62% degradation of the high M_w pectin population respectively (Table 2.4). The same level of Gal (±51 mol%, Table 2.1) was shown in both transgenic lines, but the modified pectin structures resulted in a different endoGAL digestion. The modified side chain structure of β-Gal-14 HBSS may hinder endoGAL activity, causing a lower percentage of degradation compared to that found for Karnico HBSS. The release of galactan-rich RG-I has occurred in the RGL transgenic line and caused lower residual galactan levels on the RG-I to be degraded by endoGAL. Those hypothesized pectin structures after transgenic modification will be discussed below.

Sub-populations of HBSS fractions

To investigate the possible effects of transgenic modification on the sub-populations of pectin fractions, HBSS fractions were separated according to binding of the pectin polymers present to an AEC column resulting in two sub-populations. Bound (B-fraction) and non-bound (NB-fraction) pectins were obtained. The constituent monosaccharide compositions from NB-fraction and B-fraction are shown in Table 2.5. The yield of the NB-fraction was 2× (β-Gal-14) to 3× (Karnico and RGL-18) higher than that of the B-fraction. In addition, NB-fraction comprised more galactan-rich RG-I than the B-fraction.

Both AEC sub-populations from β-Gal-14 HBSS had shorter side chains than those from Karnico, while only the B-fraction maintained the same backbone characteristics (Table 2.3). The introduction of β-Gal gene showed to shorten side chains length in sub-populations from β-Gal-14 HBSS. Fewer RG-I elements were presented (high HG:RG-I ratio), as were lower Gal:Rha ratios in both sub-populations from RGL-18 HBSS. This indicates that the introducing RGL gene resulted in a similar modification of both HBSS fractions and comparable to other pectin populations.

Table 2.5. Molar monosaccharide composition of sub-populations separated by anion exchange chromatography from hot buffer-soluble solids (HBSS) fractions of potatoes (Karnico, β-Gal-14 and RGL-18). Non-bound pectin (NB-fraction) was eluted with water; bound pectin (B-fraction) was eluted with 0.5 M sodium acetate.

Sample		Yield	Rha	Ara	Xyl	Man	Gal	Glc	GalA
		(w/w, %)				(mol%)			
Karnico	NB-fraction	75	2.6	6.7	0.3	0	78.1	3.1	9.2
	B-fraction	25	6.3	4.2	0.5	0	28.7	0.8	59.5
β-Gal-14	NB-fraction	67	5.1	6.7	0.7	0	67.7	5.1	14.7
	B-fraction	33	6.9	4.2	0.5	0	23.5	0.7	64.2
RGL-18	NB-fraction	77	3.8	5.1	0.8	0	58.7	3.9	27.7
	B-fraction	23	6.7	2.2	0.4	0	10.0	0.8	79.9

Overall effects of pectin-modifying genes on potato polysaccharides

The transgenic modifications on the pectin characteristics of the individual pectin populations, compared to that of wild-type based on the molecular structure are summarised in Table 2.6.

β-Gal-14 transgenic line

The Gal levels (mol %) and correlated Gal:Rha ratios were expected to be lower for the β-Gal transgenic line and this indeed was the case for each pectin fraction (Tables 2.1, 2.3, 2.6), except for DASS. Interactions between pectins present in DASS and other polysaccharides within the cell wall architectures might hamper the activity of β-Gal. The HBSS fraction was much more affected regarding side chain length (Gal:Rha ratio) compared to other pectin fractions. The β-Gal enzyme affected the galactan side chain of each pectin population differently. Although CWM of β-Gal-14 has lower DA and DM compared to wild-type, this was not reflected in HBSS and ChSS. HBSS pectin resulted the same levels of DA and DM, but CHSS pectin resulted higher DA and DM when compared to that of wild-type.

The hypothesized mechanism of the β-Gal enzyme modification *in vivo* in the tuber to shorten Gal side chains is validated, although the galactan side chains were modified differently to end up with different chain lengths for the various pectin populations analysed. The released Gal might be re-used by the biosynthetic machinery, but further evidence is needed. Furthermore, the side effects of introducing the β-Gal gene is influenced the pectin structure more than only side chain length and the various pectin fractions also were found to differ in esterification and pectin backbone structure.

RGL-18 transgenic line

A higher HG:RG-I ratio was seen consistently in all RGL-18 pectin fractions compared to those of the wild-type (Table 2.6). In general, the pectin fractions exhibited more pronounced effects on pectin esterification (DA and DM) and Gal:Rha ratio compared to their parental CWM (Table 2.6). We suggest that the RGL enzyme *in vivo* degraded the RG-I backbone, leaving shorter RG-I segment with less galactan side chains. Moreover, RGL is hindered by long side chains and mainly degrades RG-I backbone substituted with short side chains, and simultaneously releasing galactan-rich RG-I backbones. The released galactan-rich RG-I can explain the significant decrease of Gal in both CWM and all pectin fractions. The modified pectin backbone present in the RGL transgenic line might also be partly resulted from additional biosynthesis of HG, which may explain the higher GalA amount present (Table 2.2). The leaving galactan-rich RG-I elements might be re-used by plant itself, but more experimental research is needed to prove this hypothesis

Table 2.6. The expected and observed changes of β-galactosidase (β-Gal) or rhamnogalacturonan lyase (RGL) encoded gene on the potato transgenic lines (β-Gal-14 and RGL-18), and their effects on cell wall materials (CWM) and individual pectin fractions.

| | β-Gal | β-Gal-14 | | | | | | RGL | RGL-18 | | | | | |
| | Expected change | Observed change | | | | Sub-populations of HBSS | | Expected change | Observed change | | | | Sub-populations of HBSS | |
		CWM	HBSS	ChSS	DASS	Non-bound pectin (NB-fraction)	Bound pectin (B-fraction)		CWM	HBSS	ChSS	DASS	Non-bound pectin (NB-fraction)	Bound pectin (B-fraction)
Targeted monosaccharide	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓↓	↓↓	↓	↓↓
Non-targeted monosaccharides	–	↑↓	↑↓	↑↓	↑↓	↑↓	↑↓	–	↑↓	↑↓	↑↓	↑↓	↑↓	↑↓
Methyl-esterification	–	↓	–	↑	a	a	a	–	–	↑↑	↓	a	a	a
Acetyl-esterification	–	↓	–	↑	a	a	a	–	↓	↓↓	↓↓	a	a	a
Gal:Rha ratio (pectin side chains)	↓	↓	↓↓	↓	↑	↓↓	↓	–	↓	↓	↓↓	↓↓	↓	↓↓
HG:RG-I ratio (pectin backbone)	–	–	↓	–	–	↓	–	↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑

Abbreviation: HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids.
[a]Not determined
The values were compared with wild type Karnico based on the molar sugar composition. The symbols indicate the changes from –: 0-±10%; ↑ or ↓: ±50%;
↑↑ or ↓↓: more than ±50%; and ↑↓: both increase and decrease.

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Conclusions

After modification on pectin galactan side chains (β-Gal transgenic line) or pectin backbones (RGL transgenic line), both transgenic lines showed a lowered Gal content relatively to the Karnico control. The side effects, including those on pectin side chain (Gal:Rha ratio), pectin backbone (HG:RG-I ratio), pectic esterification (DA and DM) and other non-targeted monosaccharide in cell wall structures showed differences compared to those of the wild-type (Table 2.6). Individual pectin population showed various levels of modification, even resulting in contradicting or more significant effects when compared to CWM (Table 2.6). Side effects of the mutation showed unexpected changes for the different pectin populations. From our results, it can be concluded that the constituent monosaccharide composition of CWM might only provide an averaged overview of transgenic modifications on potato cell wall polysaccharides. Further pectin fractionation provides information on changes in individual populations upon transgenic modification.

Acknowledgements

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Chapter 3

Modification of potato tuber pectin biosynthesis also affects cell wall xyloglucan structure

Abstract

Cell wall materials were extracted from potato cultivar Karnico (wild-type) and transgenic lines carrying genes encoding for β-galactosidase (β-Gal-14 transgenic line) and rhamnogalacturonan lyase (RGL-18 transgenic line). Pectic polysaccharides from the β-Gal-14 transgenic line exhibited rhamnogalacturonan-I structural elements with shorter galactan side chains, while RGL-18 transgenic line had less rhamnogalacturonan-I structures, relatively to the wild-type. Xyloglucans from Karnico and RGL-18 1M and 4M alkali-extractable extracts were mainly of the XXGG-type, and predominantly comprised of XXGG and XSGG building blocks. Karnico and RGL-18 4M extracts have small proportions of XXXG-type xyloglucan, where in contrast, primarily the XXXG-type xyloglucan was present in the β-Gal-14 xyloglucans. The proportions of XSGG and XXGG repeating units were different between xyloglucan extracts of wild-type and two transgenic lines. So, modification of pectin also altered xyloglucan structure during plant development.

Introduction

The pectin domains from potato cell wall polysaccharides primarily include homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) (Vincken et al., 2003). These pectin structural elements are linked to each other and are bound to other polysaccharides by (non)covalent crosslinking (Cosgrove, 2005). Plant development has a strong influence on the molecular structure of these cell wall polysaccharides (Ridley et al., 2001).

The composition of potato pectin can be modified transgenically *in planta* by genes involved in pectin biosynthesis. The heterologous expression of a gene encoding β-galactosidase (β-Gal) from *Cicer arietinum* in the potato resulted in a reduction of the galactose (Gal) content of the tuber cell wall material (CWM) (Martín et al., 2005). The low Gal content is attributed to the presence of shorter galactan side chains of pectin (Huang et al., 2016). Following transgenic pectin modification, extracted pectins from the tuber differ in their arabinose (Ara), xylose (Xyl) and Gal contents (Huang et al., 2016). Expression of a rhamnogalacturonan lyase (RGL) encoding gene from *Aspergillus aculeatus* in the potato tuber resulted in removal of galactan-rich RG-I elements (Huang et al., 2016). Recent findings suggest that not only targeted structures were changed during transgenic modification, but also other cell wall polysaccharides could be modified during plant growth (Huang et al., 2016).

Potato cell walls also comprise xyloglucan, which are involved in cell growth and expansion (Kaida et al., 2010). The backbone of xyloglucan is a polymeric chain of β-(1→4)-linked d-glucopyranose units that typically contains short side chains at *O*-6. These side chains, including the substituted glucose (Glc) residue, are annotated by a single-letter code (Figure 3.1). Xyloglucans in plants can be divided into two categories according to the mean repeating units present, the so-called XXXG- and XXGG-types (Vincken et al., 1997). The *Solanaceae* family (*e.g.* potato and tomato) consists of the XXGG-type xyloglucan with varying substitutions to two out of four Glc residues throughout the xyloglucan polymer (Vincken et al., 1997).

Figure 3.1. *Nomenclature of xyloglucans (Fry et al., 1993) using a one letter code for the different side chains of xyloglucan including the backbone glucose moiety.*
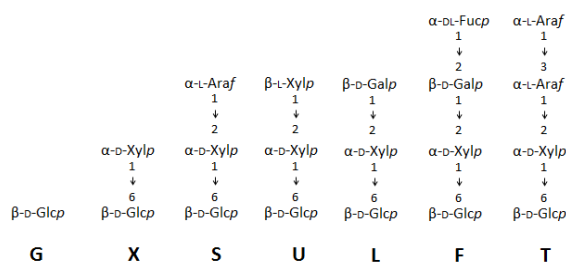
In this study, we aim to elucidate the side effects of modification in pectin biosynthesis on potato xyloglucan. Two hemicellulose fractions from wild-type and its β-Gal and RGL transgenic lines are isolated using alkali extraction after prior removal of pectic polysaccharides from potato CWM. Next, the xyloglucan structures present in these extracts are compared to that of the wild-type.

Experimental procedures

Materials

Potato transgenic lines

Transgenic potato lines expressing pectin modifying enzymes were available from previous research. The transgenes included a rhamnogalacturonan lyase (RGL-18) from *Aspergillus aculeatus* (Oomen et al., 2002) and a β-galactosidase (β-Gal-14) from *Cicer arietinum* (Martín et al., 2005). Potato tubers were grown in a greenhouse (Unifarm, Wageningen UR, Wageningen, The Netherlands), harvested (December 2011) and prepared as described previously (Huang et al., 2016).

Isolation of hemicellulose fractions from potato tubers

The potato CWM isolation procedure was described previously (Huang et al., 2016). Prior to hemicellulose extraction from CWM, pectic polysaccharides were extracted by first using a hot buffer solution (pH 5.2), followed by a chelating agent / 50 mM NaOH solution (De Vries et al., 1981). To obtain hemicellulose fractions, depectinated material was sequentially extracted using 1M and 4M sodium hydroxide each containing 20 mM NaBH$_4$ at 0°C (Vierhuis et al., 2000). Each extraction (1 h, 0°C) was performed three times. The alkali extracts and final cellulose-rich residues were neutralized using acetic acid and dialyzed against distilled water. After lyophilization, the 1M and 4M concentrated alkali-soluble solids (1M CASS and 4M CASS, respectively) were obtained.

Analytical methods

Compositions of the constituent monosaccharides

The constituent monosaccharide compositions of the CWM, 1M CASS, 4M CASS and cellulose-rich residues were analyzed using a pre-hydrolysis step with 72% (w/w) sulfuric acid at 30°C for 1 h. Subsequently, the samples were hydrolyzed with 1M sulfuric acid at 100°C for 3 h and monosaccharides released were analysed as their volatile alditol acetates by GLC as described previously (Englyst & Cummings, 1984). The total uronic acid content was determined using an automated-*m*-hydroxydiphenyl assay (Thibault, 1979). The total uronic acid content measured was attributed to GalA.

Molecular mass distributions

High-performance size exclusion chromatography (HPSEC) was performed using an Ultimate 3000 high-performance liquid chromatography system coupled to a Shodex RI-101 detector (Showa Denko, Tokyo, Japan). The HPSEC columns were TSKgel SuperAW4000, AW3000 and AW2500 columns in series and the guard column was a SuperAW-L (Tosoh Bioscience, Tokyo, Japan). The columns were kept at 55°C and 0.2 M NaNO$_3$ was used as eluent with flow rate of 0.6 mL/min. Pullulan standards (Sigma-Aldrich, St. Louis, MO, USA) were used to estimate the molecular masses of the compounds eluted.

Enzymatic degradation of the 1M and 4M CASS fractions

The 1M and 4M CASS fractions were dispersed into 50 mM sodium acetate buffer (5 mg/mL; pH 5.2). The 1M and 4M CASS fractions were digested using a xyloglucan-specific endoglucanase (XEG; EC 3.2.1.151) from *Aspergillus aculeatus* (Yaoi & Mitsuishi, 2004) and Celluclast (Novozymes, Bagsværd, Denmark), which is a cellulase preparation from *Trichoderma reesei* (Panouillé et al., 2006). Sufficient enzyme was added to degrade the corresponding substrate in 6 h at 40°C. Overnight incubation was performed to ensure complete degradation. Prior to analysis with HPSEC, supernatants were collected after centrifugation (5min, 10000 × g, 20°C).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF and MALDI-TOF/TOF mass spectra were recorded using an Ultraflextreme workstation controlled by FlexControl 3.3 software (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser of 355 nm and operated in positive mode. Xyloglucan repeating units were quantified using their relative abundances with in the mass spectrum. The sample preparation procedure for MALDI-TOF MS measurements and instrumental operation has been described elsewhere (Ramaswamy et al., 2013). For TOF/TOF fragmentation, parent and fragment ions were reaccelerated using a LIFT device located in the flight tube using the standard LIFT method optimized for the instrument. FlexAnalysis 3.3 (Bruker Daltonics) was used for data reprocessing.

Results and discussion

Constituent monosaccharide compositions of the CWM

The CWM isolated from the Karnico (wild-type), β-Gal-14 and RGL-18 consisted primarily of Gal, Glc and galacturonic acid (GalA) (Table 3.1), as already reported before (Huang et al., 2016). Compared to the wild-type, shorter RG-I galactan side chains and fewer galactan-rich RG-I elements were found in extracted pectin fractions for the β-Gal-14

and RGL-18 transgenic lines, respectively (Huang et al., 2016). The constituent monosaccharide composition of the non-extracted, hemicellulose-rich, fractions suggested that in addition to a change in pectin, other polysaccharides might have been modified as well. Consequently, in-depth analysis of the hemicellulose fraction is conducted in this research. Based on the Xyl content of the CWM fractions and assuming both XXGG- and XXXG-type xyloglucan structures to be present, it can be calculated that approximately 10 – 15% xyloglucan could be present in the CWM for the three potato varieties. The high amount of Glc might originate from residual starch.

Table 3.1. Yield, constituent monosaccharide compositions (mol%) and monosaccharide contents (mg/ 100 g tubers) in fresh tubers of CWM and corresponding 1M CASS, 4M CASS fractions and cellulose-rich residues from Karnico (wild-type) and transgenic lines (β-Gal-14 and RGL-18).

	Fractions	Yield (g/100 g CWM)	mol% (mg/100 g tubers)							Total carbohydrate content[a] (mg/ 100 g tubers)
			Rha	Ara	Xyl	Man	Gal	Glc	GalA	
Karnico	CWM[b]	-[c]	1 (15)	3 (59)	2 (33)	1 (23)	26 (605)	55 (1291)	13 (321)	66 (2347)
	1 M CASS	22.6	1 (1)	1 (4)	2 (2)	1 (5)	4 (20)	86 (443)	6 (33)	70 (513)
	4 M CASS	14.8	2 (2)	1 (1)	4 (10)	1 (1)	15 (11)	75 (62)	2 (2)	27 (90)
	Cellulose-rich residues	26.6	1 (2)	4 (7)	4 (6)	2 (4)	8 (16)	80 (163)	1 (2)	53 (201)
β-Gal-14	CWM[b]	-	1 (11)	4 (40)	2 (26)	1 (17)	23 (320)	53 (730)	16 (247)	52 (1390)
	1 M CASS	21.8	2 (5)	4 (8)	3 (6)	1 (3)	17 (44)	69 (178)	3 (14)	59 (258)
	4 M CASS	8.8	0 (0)	2 (1)	14 (6)	3 (2)	2 (1)	78 (30)	1 (1)	29 (50)
	Cellulose-rich residues	19.5	1 (1)	2 (3)	4 (10)	1 (3)	13 (39)	76 (228)	3 (10)	50 (297)
RGL-18	CWM[b]	-	1 (9)	4 (48)	3 (32)	2 (29)	14 (206)	55 (840)	22 (365)	58 (1529)
	1 M CASS	27.7	1 (3)	2 (5)	5 (12)	2 (6)	2 (6)	82 (239)	7 (22)	63 (293)
	4 M CASS	13.8	3 (1)	3 (1)	6 (5)	3 (0)	16 (5)	65 (29)	4 (2)	19 (44)
	Cellulose-rich residues	23.3	1 (2)	2 (3)	3 (5)	2 (4)	8 (16)	82 (161)	2 (4)	51 (195)

Abbreviations: CWM = cell wall materials; CASS = concentrations of alkali soluble solids
[a] Carbohydrate content of each fractions based on w/w percentage.
[b] Values of constitute monosaccharide compositions of CWMs from Karnico, β-Gal-14 and RGL-18 were reported previously (Huang et al., 2016).
[c] CWMs yields from Karnico, β-Gal-14 and RGL-18 potato tubers are 5.8%, 4.1% and 4.1% , respectively (Huang et al., 2016).

Constituent monosaccharide composition of hemicellulose extracts

1M CASS fractions

The constituent monosaccharide compositions of the 1M CASS hemicellulose fractions are presented in Table 3.1. The yields of 1M CASS were 22.6 g/ 100 g, 21.8 g/ 100 g and 27.7 g/ 100 g of CWM for Karnico, β-Gal-14 and RGL-18, respectively (Table 3.1). The total carbohydrate content expressed per 100 g fresh weight tuber of the 1M CASS fractions from β-Gal-14 and RGL-18 are 50% and 43% lower than that of the 1M CASS fraction from Karnico respectively (513 mg/ 100 g tubers, Table 3.1). Glc was the primary monosaccharide present (69 – 86 mol%) in the 1M CASS fractions (Table 3.1).

β-Gal-14 versus wild-type: The amount of Glc present in the β-Gal-14 1M fraction was 60% lower (178 mg/ 100 g tuber) compared to Karnico (443 mg/ 100 g, Table 3.1). In contrast, the Gal content was twice (44 mg/ 100 g) that of Karnico (20 mg/ 100 g tubers). Additionally, the Ara and Xyl contents (8 mg/ 100 g and 6 mg/ 100 g tubers, respectively) were higher in β-Gal-14 tubers than found for the Karnico 1M CASS fraction. The total carbohydrate content (mg/ 100 g tubers) was 50% lower for the β-Gal-14 1M CASS in comparison of wild-type. Hence, the differences in Xyl and Glc contents (mg/ 100 g tubers) might be due to a changed xyloglucan structure, since next to Xyl and Glc, also Gal and Ara might be part of the building blocks of potato xyloglucan.

RGL-18 versus wild-type: RGL-18 1M CASS exhibited a 46% decrease in Glc (239 mg/ 100 g tubers) compared to Karnico (Table 3.1). The Ara content in 1M CASS maintained at the same level: 5 mg/ 100 g *vs.* 4 mg/100 g of wild-type. The Xyl content showed a 6-fold increase for 1M CASS fraction of RGL-18 (12 mg/ 100 g tuber) compared to the Karnico 1M CASS fraction. The Gal content in 1M CASS was 70% lower (6 mg/ 100 g tuber) in comparison to that of Karnico.

4M CASS fractions

The 4M CASS fractions of Karnico and two transgenic lines yielded 8.8 – 14.8 g/100 g CWM (Table 3.1). The total carbohydrate content (mg/ 100 g tubers) present in the 4M CASS fractions of β-Gal-14 (50 mg/100 g tubers) and RGL-18 (44 mg/ 100 g tubers) were 44% and 51% lower than that of Karnico (90 mg/ 100 g tubers, Table 3.1), respectively.

β-Gal-14 versus wild-type: Both Karnico and β-Gal-14 4M CASS fractions exhibited Glc as the primary constituent monosaccharide (75 mol% and 78 mol%, respectively, Table 3.1). A possible presence of β-glucans in potato has been reported previously in 6M alkali extracts (Jarvis et al., 1981), which can explain the high contents of Glc in 4M CASS fractions. The molar proportion of other monosaccharides present in the 4M CASS fractions differed mainly between Karnico and β-Gal-14: *e.g.* Gal: 15 mol% *vs.* 2 mol% and Xyl: 4 mol% *vs.* 14 mol%, respectively (Table 3.1).

RGL-18 versus wild-type: The Glc content (mg/ 100 g tubers) in the RGL-18 4M CASS fraction (29 mg/ 100g tubers) was 53% lower compared to the Karnico 4M CASS fraction (62 mg/ 100 g tubers, Table 3.1). Karnico and RGL-18 4M CASS fractions had similar Gal molar proportions (15 mol% and 16 mol%, respectively), but the Gal content in the Karnico 4M CASS fraction was 2 times higher than that in the RGL-18 tubers (11 mg/ 100 g tubers *vs*. 5 mg/ 100 g tubers for 4M CASS fractions , respectively, Table 3.1). Considering both molar monosaccharide compositions and the fresh weight basis results of transgenic lines can provide a better evaluation of potato transgenic lines.

Cellulose-rich residues

The total carbohydrate content (297 mg/ 100 g tubers) present in the cellulose-rich residue from β-Gal-14 was 48% higher than that from Karnico (201 mg/ 100 g tubers, Table 3.1), whereas the RGL-18 residual fraction remained at the same level (195 mg/ 100 g tubers, Table 3.1) as found for Karnico.

β-Gal-14 versus wild-type: The effect on the total carbohydrate content (mg/ 100 g tubers) for the β-Gal-14 cellulose-rich residual fractions (+48%) was accompanied by a similar increase in the Glc content (mg/ 100 g tubers) (+40%). The amount of cellulose maintains the same level after introduction of β-Gal, as suggested previously (Martín et al., 2005). The change observed from the amount of Gal (+144%) is much higher than the change in total carbohydrate content (+48%) for β-Gal-14. Each monosaccharide showed to be influenced by the transgenic modification. The presence of trace amounts of GalA, Xyl and Ara might be caused by pectin or xyloglucan polysaccharides representing a stronger strength with cellulose within a modified cell wall architecture, since the xyloglucan has been proven to link to pectin polysaccharides (Zykwinska et al., 2008) or to cellulose (Lopez et al., 2010).

RGL-18 versus wild-type: Based on the similar total carbohydrate content (mg/ 100 g tubers) (-1%) and Glc content (mg/ 100 g tubers) (-3%), it can be concluded that the level of cellulose remained the same in RGL transgenic lines. The Ara and GalA contents (mg/ 100 g tubers) differed with those of the wild-type (-57% and +100% respectively). The modified pectin structure with less galactan-rich RG-I structure elements influenced the cell wall architecture of RGL transgenic line.

Interactions within the cell wall architecture

Based on the constituent monosaccharide compositions of the hemicellulose fractions, a modified xyloglucan structure could be a response of the plant to compensate for the effect on galactan side chains in the pectin. Zykwinska et al. (2005) reported that the neutral side chains of pectins can bind to cellulose. β-Gal transgenic lines had shorter galactan side chains of pectin (Huang et al., 2016). These shorter galactan side chains might interact differently with cellulose, which could require a different xyloglucan structure to maintain

the strength. The RGL transgenic lines had less galactan-rich RG-I segments within its pectin structures (Huang et al., 2016), which might result in less interaction of pectic galactan to the xyloglucan-cellulose network.

Molecular weight distribution of the hemicellulose fractions and their enzyme digests

The hemicellulose fractions obtained from the 1M and 4M CASS fractions were investigated degradation by XEG alone or XEG in combination with Celluclast.

1M CASS fractions and enzymatic degradation

All three 1M CASS fractions contained carbohydrates with M_w ranging between ±1 to ±150 kDa. Whereas Karnico (Figure 3.2A) and RGL-18 (Figure 3.2C) showed a predominant population eluting around 5 kDa, RGL-18 had more carbohydrate polymers distributed at range of ±3 to 18 kDa than Karnico. RGL-18 had a minor population with a Mw of around 66 kDa. β-Gal-14 (Figure 3.2B) had a more broad distribution of molecular weights, including a main population in the range of ±2 to ±20 kDa. Following digestion by XEG, the M_w distribution shifted slightly to lower M_w. Ramasamy et al. (2014) used Celluclast for an improved degradation of complex xyloglucan polymers. Whereas the combination of XEG and Celluclast resulted in a similar degradation pattern for the Karnico 1M CASS fraction (Figure 3.2A) as incubation XEG alone, more degradation was obtained for the mutant fractions (Figures 3.2B and 3.2C).

4M CASS fractions and enzymatic degradation

The 4M CASS fraction of both Karnico (Figure 3.3A) and RGL-18 (Figure 3.3C) had a M_w distribution ranging from ± 1.5 to ±70 kDa. The main populations were around 5 kDa for both Karnico and RGL-18, but RGL-18 had broader peak distribution than Karnico (Figures 3.3A and 3.3C). Compared to the Karnico and RGL-18 4M CASS fractions, the β-Gal-14 4M CASS fraction was less soluble (Figure 3.3B). The RGL-18 4M CASS could be substantially more degraded by XEG than the Karnico fraction. By adding Celluclast to XEG, the degradation of both the Karnico and RGL-18 fractions increased (Figures 3.3A and 3.3C). The β-Gal-14 4M CASS fraction degraded by XEG + Celluclast may have released Glc monomer or dimer, which eluted after ±1.5 k Da, but could not be recognized due to the presence of the buffer peak (data not shown).
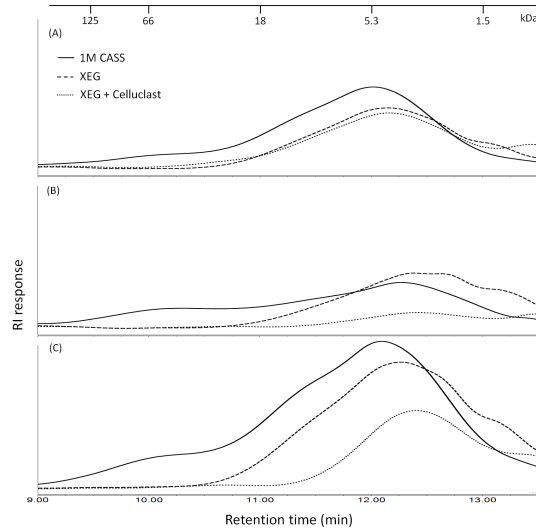
Figure 3.2. HPSEC chromatograms of (A) Karnico 1 M concentration of alkali soluble solids (CASS); (B) β-Gal-14 1 M CASS; and (C) RGL-18 1 M CASS and digests obtained using xyloglucan-specific endoglucanase (XEG) alone and XEG in combination with Celluclast. Molecular masses of pullulan standards (in kDa) are indicated.
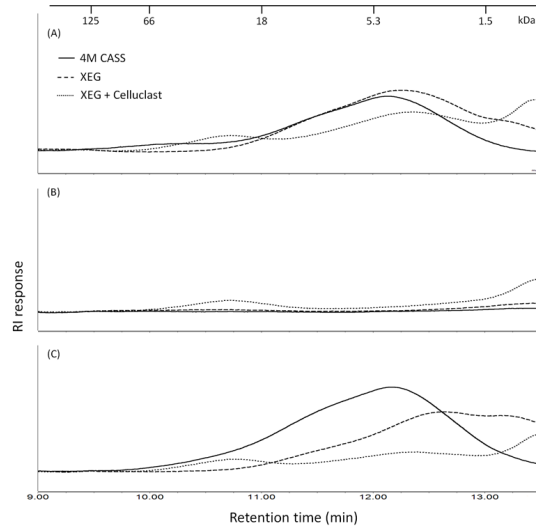
Figure 3.3. HPSEC chromatograms of (A) Karnico 4 M concentration of alkali soluble solids (CASS); (B) β-Gal-14 4 M CASS; and (C) RGL-18 4 M CASS and digests obtained using xyloglucan-specific endoglucanase (XEG) alone and XEG in combination with Celluclast. Molecular masses of pullulan standards (in kDa) are indicated.

Xyloglucan oligomer profiling

The 1M CASS and 4M CASS fractions could not be degraded by XEG sufficiently and provided complex mass spectra (data not shown), which could not be easily identified. The combination of XEG and Celluclast provided better degradation of xyloglucan polymers than using XEG alone and results are discussed below.

1M CASS fractions digested by XEG + Celluclast

MALDI-TOF MS was used to identify the xyloglucan oligomers XSG (*m/z* 923) and XXG (*m/z* 791) were the dominant structures identified in Karnico 1M CASS digest (Figure 3.4A). The Celluclast is reported to degrade the xyloglucan backbone in repeating units and is also able to cleave off the terminal Glc on the reducing or non-reducing end (Ramasamy et al., 2014). Therefore, we suggested that XXG and XSG are derived from the xyloglucan repeating units XXGG (*m/z* 953) and XSGG (*m/z* 1085). Potato xyloglucan oligomers are built up by XXGG-type xyloglucan, including XXGG, XSGG, XLGG and LSGG repeating units (Vincken et al., 1996), which is consistent with our identification of XSGG and XXGG. XLGG and LSGG repeating units have been found in cultivar Bintje (Vincken et al., 1996), but were not identified in Karnico as well as in its two transgenic lines.

The β-Gal-14 and RGL-18 1M CASS fractions degraded by XEG + Celluclast had the same dominant peaks in the corresponding mass spectra, but the peaks showed different relative abundances. The ratio (XSGG + XSG)/ (XXGG + XXG) was calculated based on the intensity of corresponding mass peaks of mass spectrum and resulted in values of 3.2, 4.4 and 3.5 for Karnico, RGL-18 and β-Gal-14 1M CASS fractions, respectively. RGL-18 xyloglucan seems to have a lower proportion of XXGG repeating units. Xyloglucan has been reported to non-covalent adsorb to cellulose fibrils (Rizk et al., 2000). The decoration of xyloglucan side chains (e.g., XSGG or XXGG) may change the conformation of xyloglucan through the folding of the xyloglucan side chain along the backbone (Zhang et al., 2011). The modified conformation influences the interaction between xyloglucan and cellulose, which probably contributes to a stable cell wall.

The mass spectrum of the xyloglucan digest had a peak *m/z* 1055 (Figure 3.4B) corresponded to 3Hexose (H) + 4Pentose (P). The *m/z* 761 (loss 1P + 1H) indicated the possible cleavage of 1→4 linked Glc on the backbone, which might have a pentose linked to Glc residues. The fragment *m/z* 671 was caused by the loss of 90 Da ($C_3O_3H_6$) from *m/z* 761 (Figure 3.4B) indicating the presence of an 1→4 linkage between hexoses at the reducing end of the molecule, as found previously (Bauer, 2012). These identifications lead us to propose the XTG structure (3P + 1H, Figure 3.4B). Based on the mass fragmentation spectrum and available studies, the *m/z* 1055 may refer to XTG, which has been reported in tomato (*Solanum lycopersicum*) (Jia et al., 2003), being the same Solanaceae family as

potato. XTG (*m/z* 1055) is considered to originate from XTGG (*m/z* 1217) upon removal of a terminal glucosyl residue (-162Da) by XEG + Celluclast. The trace amounts of *m/z* 1379 (XTGG + H) and *m/z* 1247 (XSGG + H) might correspond to GXTGG and GXSGG as the most possible structures. Similar structures (GLLGG and GSSGG) were reported in potato cultivar Bintje (Vincken et al., 1996). Although, *m/z* 1379 (XTGG + H) and *m/z* 1247 (XSGG + H) might refer to XTGGG and XSGGG as well. The low intensity of *m/z* 1349 (XSGG + H + P) was insufficient for MALDI-TOF/TOF MS interpretation.

A hexose oligomeric series (Figure 3.4A: H$_n$; n = 4−13) was observed, which might be residual starch still present in the 1M CASS fractions.
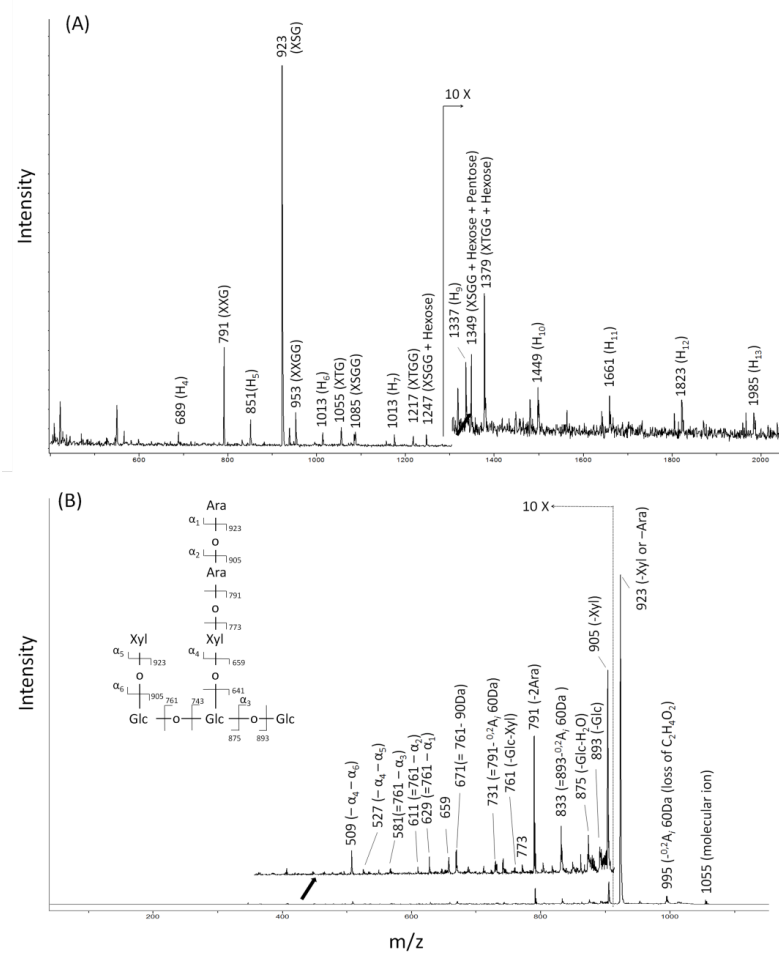
Figure 3.4. (A) MALDI-TOF mass spectrum of 1 M concentration of alkali soluble solids (CASS) of Karnico and (B) MALDI-TOF/TOF MS mass spectra of the peak at *m/z* 1055 from 1 M CASS from Karnico digested using xyloglucan-specific endoglucanase (XEG) in combination with Celluclast.

47

4M CASS fractions digested by XEG + Celluclast

The mass spectra of the Karnico and RGL-18 4M CASS digests exhibited the same two series of *m/z* values representing xyloglucan fragments and hexose oligomers (Figures 3.5A and 3.5C, respectively). The peaks are summarized in Figure 3.5D. The peak ratios of (XSGG + XSG) / (XXGG + XXG) are 1.9 and 2.4 for Karnico and RGL-18, respectively. The 4M CASS xyloglucan from RGL-18 had more XSGG repeating units than those of Karnico.

The peaks at *m/z* 527, *m/z* 689, *m/z* 851, *m/z* 1013, *m/z* 1175, etc. could be attributed to hexose oligomers (H_{3-19} in Figure 3.5). The hexose oligomers present might originate from the presence of β-glucans from 4M CASS fractions. β-Glucans have been reported previously to be present in 4M CASS extracts of potato fiber (Ramaswamy et al., 2013) or 6M NaOH extracts of potato (Jarvis et al., 1981). Some peaks, such as *m/z* 730, *m/z* 892 and *m/z* 939, could not be identified.

No.	m/z	Symbol
1	527	H_3
2	689	H_4
3	730	unknown
4	791	XXG
5	851	H_6
6	892	unknown
7	923	XSG
8	939	unknown
9	953	XXGG
10	1013	H_7
11	1055	XTG
12	1085	XSGG
13	1175	H_8
14	1217	XTGG
15	1337	H_9
16	1379	XTGG + hexose
17	1499	H_{10}
18	1541	LLUG
19	1661	H_{11}
20	1703	LLUG + hexose
*		H_2O adduction
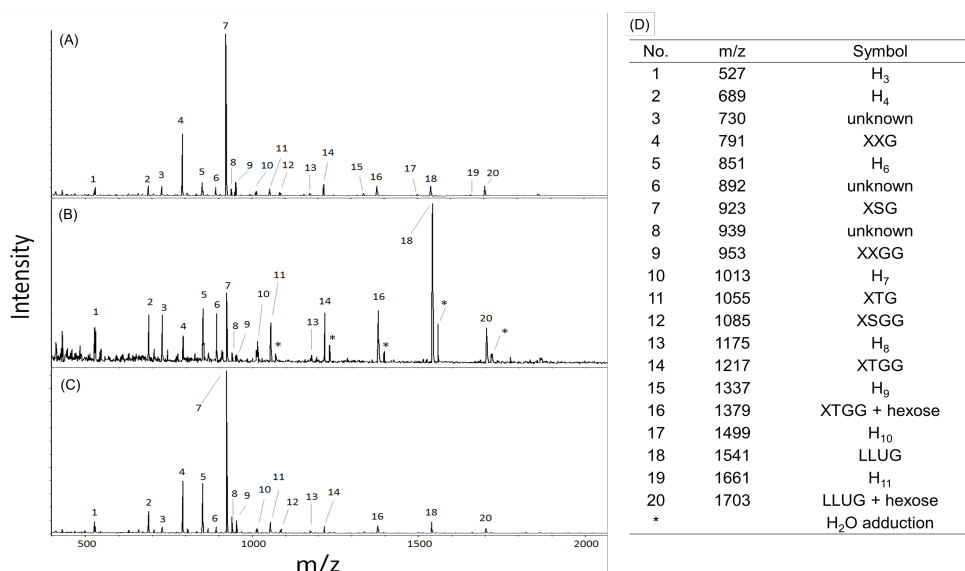
Figure 3.5. MALDI-TOF mass spectra of 4 M concentration of alkali soluble solids (CASS) of (A) Karnico, (B) β-Gal-14, and (C) RGL-18 β-Gal digested using xyloglucan-specific endoglucanase (XEG) in combination with Celluclast. (D) List of peaks numbers from 1 to 20 represented the corresponding *m/z* of hexose series (H_n), xyloglucan repeating units and some the xyloglucan repeating units the with the H_2O (+ 18 Da) adduction.

The *m/z* 1541 is a minor peak in Karnico (6 H + 4 P), but not for β-Gal-14. The *m/z* 953 peak ([M] – 2 H – 2 P) might indicate a cleavage between the second and third Glc residues ($β_{13}$ cleavage, Figure 3.6). The peak *m/z* 1247 (loss of P and H) indicated that Gal and Xyl

might be linked to Glc residues (Figure 3.6). The loss of two pentose from the side chain part yields the *m/z* 1277 (Figure 3.6). Internal cleavage by a loss of 60 Da or 120 Da was observed in the *m/z* 1541 fragment (Figure 3.6): *e.g.*, *m/z* 1481 (*m/z* 1541 – 60 Da), *m/z* 833 (*m/z* 953 – 120 Da). The *m/z* 326, *m/z* 1023, *m/z* 1073 and *m/z* 1440 peaks (Figure 3.6) could not be explained by this study. The *m/z* 1055, *m/z* 1217 and *m/z* 1541 had a H_2O (+ 18 Da) adduction (Figure 3.5B). LLUG might be the possible xyloglucan repeating unit for *m/z* 1541. LLUG has not been identified before for potato, but has been found before in the XXXG-type xyloglucan from bilberry (Hilz et al., 2007). β-Gal-14 4M CASS xyloglucan has predominant LLUG repeating units. In addition, the ratios (XSGG + XSG) / (XXGG + XXG) are 1.1 and 1.9 for β-Gal-14 and Karnico, respectively. Xyloglucan contributes to the rigidity of the cell wall when it cross-links adjacent cellulose microfibrils (Hayashi, 1989). After modification of galactan side chains in β-Gal transgenic lines, a changed xyloglucan structure might have been biosynthetically modified during plant development. The unexpected changes in xyloglucan structure might provide strength for cell wall architecture by plant biosynthesis, but more evidence is needed.
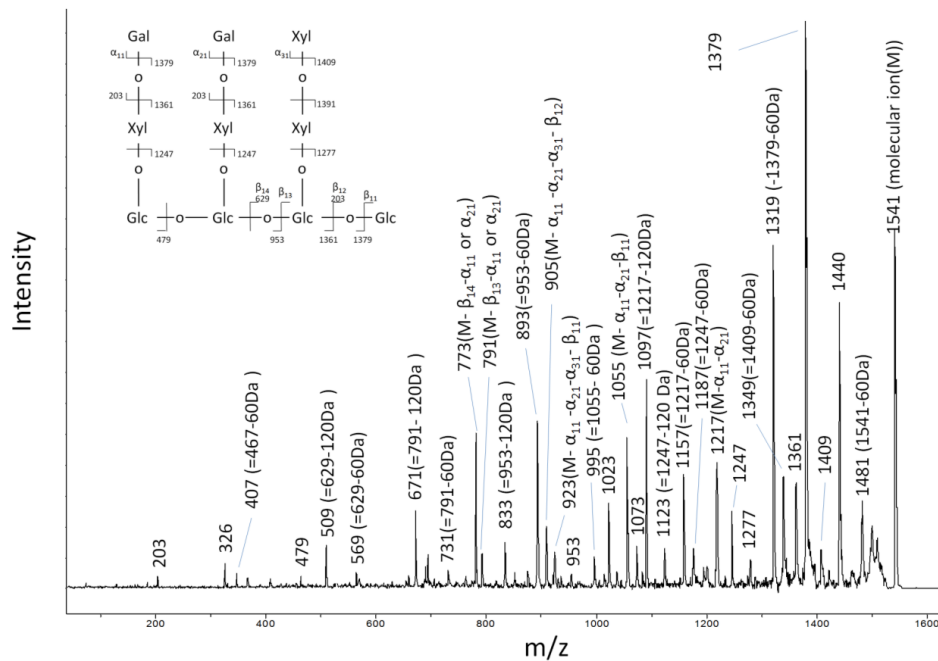
Figure 3.6. MALDI-TOF/TOF mass spectra of the peak at *m/z* 1541 of β-Gal-14 4 M concentration of alkali soluble solids (CASS) digested using xyloglucan-specific endoglucanase (XEG) in combination with Celluclast.

Conclusions

In this study, RGL transgenic lines have predominantly XXGG-type xyloglucan structures as can be seen from the Karnico. β-Gal transgenic lines have predominantly XXXG-type xyloglucans. Both RGL and β-Gal transgenic lines have different proportions of xyloglucan building blocks in comparison to wild-type. Transgenic modification on pectin side chain or pectin backbone clearly affects the xyloglucan structure.

Acknowledgements

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Chapter 4

Alteration of cell wall polysaccharides through expression of UDP-Glc 4-epimerase-encoding genes in potato tubers

Abstract

Uridine diphosphate (UDP)-glucose 4-epimerase (UGE) catalyzes the conversion of UDP-glucose to UDP-galactose. Cell wall materials from the Kardal (wild-type) and the UGE 45-1 and UGE 51-16 transgenic lines were isolated and fractionated. The galactose (Gal) content (mg/100g tuber) from UGE 45-1 transgenic line was 38% higher than that of wild-type, and resulted in longer pectin side chains. The Gal content present in UGE 51-16 was 17% lower than that of wild-type, although most pectin populations maintained the same level of Gal. Both UGE transgenic lines showed unexpectedly a decrease in acetylation and an increase in methyl-esterification of pectin. Both UGE transgenic lines showed similar proportions of homogalacturonan and rhamnogalacturonan I within the pectin backbone as the wild-type, except for the calcium-bound pectin fraction exhibiting relatively less rhamnogalacturonan I. Next to pectin modification, xyloglucan populations from both transgenic lines were altered resulting in different XSGG and XXGG proportion in comparison to wild-type.

Introduction

Pectin consists primarily of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). HG is an unbranched polymer of α-(1→4)-linked galacturonosyl residues, whereas the backbone of RG-I is composed of repeating α-(1→2)-L-rhamnose (Rha)-α-(1→4)-D-galacturonic acid (GalA) units with side chains connected to the rhamnose moiety (McCann & Roberts, 1991). Potato pectin is rich in galactan side chains consisting of (1→4)-β-D-galactose (Gal) residues connected to the O-4 position of Rha within the RG-I backbone (Sørensen et al., 2000).

The primary hemicellulose found in potato tubers is xyloglucan (Vincken et al., 2000). Xyloglucan is a polysaccharide found in primary cell walls and plays a role in the control of cell growth (Fry, 1989). Xyloglucan consists of a β-(1→4)-D-glucan backbone and is substituted with rather specific short side chains of xylose with or without additional substitution of Gal, fucose and/or arabinose (Ara). These side chains including the substituted glucose (Glc) moiety are individually annotated using a single letter code (Fry et al., 1993). In *Solanaceae* (e.g., potato), the xyloglucan consists of XXGG-type repeating units (RU), in which two out of four Glc residues in the backbone are substituted at O-6 with α-d-Xyl residues which may have additional monosaccharides substitution attached (Vincken et al., 1996).

The composition of potato pectin can be biosynthetically modified to alter cell wall architecture. Caffall and Mohnen (2009) reported that cell wall biosynthesis requires the expression of glycosyltransferases that utilize uridine diphosphate (UDP) sugars for the assembly of cell wall polysaccharides. They further discovered that pectin biosynthesis was directly affected by changes in the nucleotide sugar pools. Nucleotide sugars are synthesized by various types of interconverting enzymes, such as epimerases, decarboxylases and dehydrogenases, and their individual levels in the sugar pool directly affect the content of the corresponding monosaccharides in cell wall polysaccharides (Gondolf et al., 2014). The pools of UDP-sugar are directly or indirectly linked by either one-way or reversible reactions to form other UDP-sugars (Reiter & Vanzin, 2001). The Glc:Gal ratio can be influenced by UDP-glucose-4-epimerase (UGE), which catalyzes a reversible conversion of UDP-glucose (UDP-Glc) into UDP-galactose (UDP-Gal) as demonstrated in Arabidopsis mutants (Bar-Peled & O'Neill, 2011). The introduction of the gene from Arabidopsis that encodes UGE in potato increased the Gal levels in potato cell walls (Oomen et al., 2004). Oomen et al. (2004) focused primarily on the Gal levels in the cell wall materials (CWM) of UGE transgenic lines and hardly investigated the consequences of these changed levels for polysaccharide structures. Recently, we reported the appearance of shorter galactan side chains present in potato pectin after the introduction of the β-galactosidase (β-Gal) gene into wild-type potato (Huang et al., 2016). In contrast to the wild-type, the β-Gal transgenic lines predominantly contained XXXG-type xyloglucan

in their cell walls as a side effect (Chapter 3). In UGE transgenic lines, the precise mechanism of both targeted and non-targeted modifications of the cell wall polysaccharides remained unclear.

In the present study, we characterized the structure of pectins found in UGE transgenic lines and wild-type potato, and identified additional changes within other polysaccharides.

Methods

Experimental procedures

Potato tubers

Two different genes, UGE 45 and UGE 51, were used for the preparation of a sense construct using the pBIN20 vector with wild-type potato Kardal as the background to obtain UGE 45-1 and UGE 51-16 transgenic lines as described before (Oomen et al., 2004). Both UGE 45 and UGE 51 genes encoded the UGE enzyme (Oomen et al., 2004). Kardal, UGE 45-1 and UGE 51-16 were grown in a greenhouse (Unifarm, Wageningen UR, Wageningen, The Netherlands) and harvested in December 2011. Potato tubers were peeled and diced into 5-mm cubes, which were then directly frozen in liquid nitrogen. The samples were stored at −20°C until use.

Isolation of CWM

CWM was obtained after an extensive starch removal step as described elsewhere (Huang et al., 2016).

Sequential extraction of CWM

The potato CWM were sequentially extracted as described elsewhere (Vierhuis et al., 2000). In short, hot buffer-soluble solids (HBSS) were extracted using 0.05 M sodium acetate buffer (pH 5.2) at 70°C; chelating agent-soluble solids (ChSS) were extracted using 0.05 M EDTA and 0.05 M sodium oxalate in 0.05 M sodium acetate buffer (pH 5.2) at 70°C; diluted alkali-soluble solids (DASS) were extracted using 0.05 M sodium hydroxide containing 0.02 M sodium borohydride at 0°C; and 1M and 4M concentrated alkali soluble solids (1M CASS and 4M CASS) were extracted using 1M and 4M sodium hydroxide containing 0.02 M sodium borohydride at 0°C. Fractions containing ChSS were subsequently dialyzed against ammonium acetate buffer (0.1 M, pH 5.2) for 18 hours and distilled water, and freeze-dried. Other extracts were dialyzed directly against distilled water and freeze-dried. The final residues were washed with distilled water and freeze-dried.

Analytical methods

Constituent monosaccharide composition

The constituent monosaccharide compositions of the CWM and polysaccharide fractions were analyzed using a pre-hydrolysis step with 72% (w/w) sulfuric acid at 30°C for 1 h. Subsequently, the samples were hydrolyzed with 1M sulfuric acid at 100°C for 3 h and monosaccharides released were analysed as their alditol acetates by GLC (Englyst & Cummings, 1984). The total uronic acid content was determined using an automated-m-hydroxydiphenyl assay (Thibault, 1979). The total uronic acid content measured was attributed to GalA.

The starch content of CWM was analyzed enzymatically using the Megazyme Total Starch Kit (Megazyme, Wicklow, Ireland). Starch Glc was subtracted from total Glc to obtain cell wall Glc.

Using the constituent molar monosaccharide composition, the average length of the galactan side chain is described using the molar ratio of Gal:Rha. The ratios of the backbone structural elements HG and RG-I are described by the HG:RG-I ratio, where HG = [GalA] – [Rha], and RG-I = 2 × [Rha], respectively. [GalA] and [Rha] are the molar proportions of GalA and Rha, respectively.

Degree of acetylation (DA) and methyl-esterification (DM)

The acetylation and methyl-esterification present in the polysaccharides were determined by HPLC after alkali treatment as described previously (Voragen et al., 1986). They are calculated as mole methyl esters or acetyl groups per 100 mole GalA, respectively. One mole GalA can carry only one mole methyl esters and two moles of acetyl groups.

Molecular mass distribution

The molecular mass distribution of the extracted pectin fractions was analyzed using high performance size exclusion chromatography (HPSEC) on an Ultimate 3000 HPLC system coupled to a Shodex RI-101 detector (Showa Denko, Tokyo, Japan). The HPSEC columns used were TSK gel Super AW4000, AW3000 and AW2500 columns in series, and the guard column was a Super AW-L (Tosoh Bioscience, Tokyo, Japan). The columns were kept at 55°C. The eluent was 0.2 M $NaNO_3$ using a flow rate of 0.6 mL/min. Pullulan standards (Sigma-Aldrich, St. Louis, MO, USA) were used to estimate the molecular mass of the eluted compounds.

Degradation levels of high molecular weight (M_w) pectin population were calculated in the range of 7.0-10.5 min (±40 −300 kDa). The level of degradation was defined as area of 7.0-10.5 min after enzymatic degradation / area of 7.0-10.5 min before enzymatic degradation.

Enzymatic degradation of polysaccharide fractions

HBSS fractions were dispersed (5 mg/mL) in 50 mM sodium acetate buffer (pH 5.2) and subsequently digested at 40°C using endopolygalacturonase (PG; EC 3.2.1.15) from *Aspergillus aculeatus* (Limberg et al., 2000), pectin methylesterase (PME; EC 3.1.1.11) from *Aspergillus niger* (Van Alebeek et al., 2003), endo-β-1,4-galactanase (endoGal; EC 3.2.1.89) from *Aspergillus niger* (Van de Vis et al., 1991) and β-1,4-galactosidase (exoGal; EC 3.2.1.23) from *Aspergillus niger* (Van de Vis, 1994).

The 1M and 4M CASS fractions were dispersed (5 mg/mL) in 50 mM sodium acetate buffer (pH 5.2) and subsequently digested at 40°C using xyloglucan-specific endo-β-1,4-glucanase (XEG; EC 3.2.1.151) from *Aspergillus aculeatus* (Yaoi & Mitsuishi, 2004) and Celluclast (Novozymes, Copenhagen, Denmark), which is a cellulase preparation from *Trichoderma reesei* (Panouillé et al., 2006). Sufficient enzyme was added to degrade the corresponding substrate in 6 hours and the fractions were incubated overnight for complete degradation. Prior to analysis by HPSEC, supernatants were collected after centrifugation (5 min, 10000 × g, 20°C).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS spectra were recorded using an Ultraflextreme workstation controlled by FlexControl 3.3 software (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser of 355 nm and operated in positive mode. MS was used to quantify the digested polysaccharides. The sample preparation procedure for MALDI-TOF MS measurements and instrumental operation has been described elsewhere (Ramaswamy et al., 2013).

Results

CWM composition of wild-type potato and transgenic lines

The starch levels in the isolated CWM from Kardal, UGE 45-1 and UGE 51-16 were 5.1%, 4.5% and 4.1% of CWM, respectively. The constituent molar monosaccharide composition of the polysaccharides in each CWM (Table 4.1) and the contents of constituent monosaccharides in each CWM, expressed on fresh tuber weight basis (mg/100 g tuber, Table 4.2) revealed distinct differences in the cell wall composition between Kardal and UGE 45-1 and UGE 51-16 transgenic lines. The amount of carbohydrates was rather similar for all potato varieties (±3000 mg/100 g tuber, Table 4.2). Glc, Gal and GalA were the primary monosaccharides (Tables 4.1 and 4.2). A higher Gal content (1279.8 mg/100 g tuber) was observed in UGE 45-1 than in Kardal (926.7 mg/100 g tuber, Table 4.2). In contrast, the Gal content of UGE 51-16 was lower (767.4 mg/100 g tuber, Table 4.2). Rha,

Ara, xylose (Xyl) and mannose (Man) were present in low quantities in all potato varieties (Tables 4.1 and 4.2).

The cell wall pectin from UGE transgenic lines exhibited increased methyl-esterification (DM = 44 and 43 for UGE 45-1 and UGE 51-16, CWMs respectively), whereas the acetylation level was slightly decreased (DA = 20 and 23 for UGE 45-1 and UGE 51-16 CWMs, respectively) when compared to the values found for wild-type (DM = 35; DA = 26, Table 4.1).

Table 4.1. Molar monosaccharide composition (mol %) and degree of acetylation (DA) and methyl-esterification (DM) of cell wall material and its fractions of Kardal (wild-type) and transgenic UGE 45-1 and UGE 51-16 potato tubers.

Name	mol %							Carbohydrate content (%)[a]	DM (%)[b]	DA (%)[b]
	Rha	Ara	Xyl	Man	Gal	Glc	GalA			
Kardal CWM	0.9	3.9	2.7	1.1	31.0	52.4[c]	8.0	62.7	35	26
Kardal HBSS	1.3	6.5	0.8	0.3	77.7	2.5	10.9	76.5	16	33
Kardal ChSS	0.5	6.2	0.0	1.5	20.5	15.0	56.3	27.2	36	2
Kardal DASS	0.5	4.0	0.0	1.8	14.1	75.7	3.9	21.5	-[d]	-
Kardal 1M CASS	0.5	3.3	7.9	0.8	8.6	77.3	1.6	79.7	-	-
Kardal 4M CASS	0.3	4.0	9.1	4.2	7.8	73.2	1.4	56.9	-	-
Kardal residues	0.3	2.0	1.3	0.4	0.9	93.5	1.6	71.6	-	-
UGE 45-1 CWM	0.7	3.3	2.6	0.9	41.4	44.6[c]	6.5	60.9	44	20
UGE 45-1 HBSS	1.0	5.3	0.8	0.3	79.7	3.8	9.1	78.3	17	27
UGE 45-1 ChSS	0.6	9.3	0.0	0.8	27.9	10.8	50.6	22.9	55	2
UGE 45-1 DASS	0.5	4.0	0.0	2.1	19.4	70.6	3.4	20.1	-	-
UGE 45-1 1M CASS	0.5	4.0	10.3	1.0	12.4	70.1	1.7	71.0	-	-
UGE 45-1 4M CASS	0.3	2.5	5.6	3.1	5.0	82.4	1.1	75.5	-	-
UGE 45-1 residues	0.3	2.0	1.3	0.3	1.6	93.0	1.5	59.8	-	-
UGE 51-16 CWM	1.0	4.2	3.3	1.2	24.7	56.1[c]	9.5	61.1	43	23
UGE 51-16 HBSS	1.4	6.5	0.8	0.3	73.7	3.1	14.2	63.2	19	31
UGE 51-16 ChSS	0.8	5.9	0.0	0.6	18.5	5.9	68.3	27.8	33	2
UGE 51-16 DASS	0.5	4.4	0.0	3.8	12.1	74.0	5.2	11.6	-	-
UGE 51-16 1M CASS	0.6	3.1	7.6	0.9	8.6	77.5	1.7	67.2	-	-
UGE 51-16 4M CASS	0.4	5.2	11.6	4.3	9.6	67.0	1.9	39.3	-	-
UGE 51-16 residues	0.4	2.0	1.3	0.3	0.8	93.8	1.4	71.1	-	-

Abbreviations: CWM = cell wall materials; HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids; CASS = concentrations of alkali soluble solids.
Starch levels from Kardal CWM, UGE45-1 CWM and UGE 51-16 were 5.3%, 4.5% and 4.1%, respectively.
[a]Carbohydrate content based on % w/w of CWM.
[b]Number of moles of acetyl or methyl groups per 100 mol of galacturonic acid.
[c]Glc content after starch correction.
[d]Not determined.

Table 4.2. Yield and constituent cell wall monosaccharide composition of cell wall material (CWM) and its fractions from Kardal (wild-type), UGE 45-1 and UGE 51-16 potato based on fresh potato tuber basis.

Name	Yield [g/100 g CWM]	mg/100 g tuber							Amount of carbohydrate
		Rha	Ara	Xyl	Man	Gal	Glc	GalA	
Kardal CWM		23.6	97.9	68.3	32.3	926.7	1565.7[a]	256.4	2970.7
Kardal HBSS	28.6	10.4	46.1	5.4	3.0	661.8	20.9	99.6	847.2
Kardal ChSS	8.0	1.1	12.0	0.0	3.4	47.3	34.5	139.6	237.8
Kardal DASS	26.5	3.8	26.7	0.0	14.3	111.4	599.4	33.1	788.7
Kardal 1M CASS	12.2	1.8	10.1	24.2	3.1	31.7	284.1	6.5	361.4
Kardal 4M CASS	6.3	0.5	6.5	14.5	8.0	15.0	140.2	3.0	187.6
Kardal residues	10.7	1.0	5.4	3.4	1.2	2.8	297.8	5.5	317.1
UGE 45-1 CWM		20.9	85.7	66.7	27.3	1279.8	1375.6[a]	216.8	3072.8
UGE 45-1 HBSS	30.5	9.0	42.8	6.5	2.5	766.7	36.1	94.8	958.4
UGE 45-1 ChSS	8.1	1.4	19.2	0.0	1.9	69.2	26.9	135.3	254.0
UGE 45-1 DASS	31.7	4.3	32.9	0.0	20.8	193.2	702.1	36.4	989.6
UGE 45-1 1M CASS	8.8	1.3	9.5	24.2	2.9	35.0	198.3	5.1	276.4
UGE 45-1 4M CASS	3.9	0.3	2.6	5.8	3.9	6.2	102.9	1.5	123.3
UGE 45-1 residues	8.2	0.8	4.2	2.7	0.7	4.0	239.3	4.1	255.9
UGE 51-16 CWM		29.2	108.1	85.1	36.3	767.4	1749.7[a]	317.5	3093.5
UGE 51-16 HBSS	31.1	11.5	48.6	6.2	2.7	656.7	27.8	136.6	890.1
UGE 51-16 ChSS	6.9	1.5	10.2	0.0	1.2	38.2	12.2	152.0	215.2
UGE 51-16 DASS	35.7	5.1	41.1	0.0	42.6	135.5	829.3	62.4	1116.0
UGE 51-16 1M CASS	10.7	1.8	8.9	21.5	2.9	29.2	263.9	6.2	334.4
UGE 51-16 4M CASS	6.4	0.7	8.9	19.8	8.8	19.6	136.8	4.2	198.8
UGE 51-16 residues	8.7	0.9	4.5	3.0	0.9	2.2	255.9	4.0	271.4

Abbreviations: HBSS = hot buffer-soluble solids; ChSS = chelating agent-soluble solids; DASS = diluted alkali-soluble solids; CASS = concentrations of alkali soluble solids.
[a] Glc content after starch correction.

Different polysaccharide populations

To understand effects in the different polysaccharide populations present in the CWM, pectin and hemicellulose were sequentially extracted from the CWMs. Individual pectin (HBSS, ChSS and DASS) fractions, hemicellulose (1M CASS and 4M CASS) fractions and residues were analyzed for their constituent monosaccharide composition (Tables 4.1 and 4.2). The recovery of the fractionation on weight base was 92%, 93% and 98% for Kardal, UGE 45-1 and UGE51-16, respectively.

The yield of the *HBSS fractions* varied between 28.6-31.1 (g/100 g CWM, Table 4.2). All fractions revealed Gal and GalA as the main sugars (Tables 4.1 and 4.2). Compared to Kardal HBSS, UGE 45-1 HBSS had a higher Gal content (766.7 mg/100 g tuber vs. 661.8 mg/100 g tuber for Kardal HBSS) and a similar GalA content (94.8 mg/100 g tuber vs. 99.6 mg/100 g tuber, Table 4.2). UGE 51-16 HBSS showed a similar Gal content (656.7 mg/100 g tuber), but a higher GalA content (136.6 mg/100 g tuber) compared to Kardal (Table 4.2). The DA and DM of the Kardal HBSS fraction were 33% and 16%, respectively (Table 4.1). Compared to wild-type Kardal, UGE 45-1 HBSS had lower acetylation of pectin (DA = 27)

but maintained a similar level of methyl-esterification (DM = 17, Table 4.1). A slightly higher methyl-esterification (DM = 19) was found in UGE 51-16 HBSS, while the acetylation of the pectin remained at the same level in comparison to the wild-type (DA = 31, Table 4.1).

ChSS fractions showed a low yield: 6.9-8.1 g/100 g CWM (Table 4.2). GalA was the most abundant monosaccharide in the ChSS fractions and ranged from 135.3 to 152.0 mg/100 g tuber (Table 4.2). A higher (69.2 mg/100 g tuber) and lower (38.2 mg/100 g tuber) Gal content for UGE 45-1 and UGE 51-16, respectively, in comparison to the wild-type (47.3 mg/100g tuber, Table 4.2). All ChSS fractions were low in their acetylation (DA = 2, Table 4.2). UGE 45-1 ChSS pectin consisted of relatively high methyl-esterified pectin (DM = 55) when compared to wild-type (DM = 36) and UGE 51-16 ChSS (DM = 33, Table 4.2).

DASS fractions yielded from 26.5 to 35.7 g per 100 g of CWM (Table 4.2). Glc was the primary monosaccharide in this fraction. Both mutants had higher Glc content (702.1 and 829.3 mg/100 g tuber for UGE 45-1 DASS and UGE 51-16 DASS, respectively) than wild-type (599.4 mg/100 g tuber, Table 4.2). The Gal contents were higher in UGE transgenic lines (193.2 and 135.5 mg/ 100 g tuber for UGE 45-1 and UGE 51-16, respectively, Table 4.2) compared to wild type (111.4 mg/100 g tuber).

The yield of *1M CASS fractions* ranged between 8.8-12.2 g/100 g CWM (Table 4.2). All potato varieties had Glc as the dominant monosaccharide in this fraction (Table 4.1). Kardal and UGE 51-16 1M CASS had the same levels of Glc (284.1 and 263.9 mg/100 g tuber, respectively), being higher than that of UGE 45-1 (198.3 mg/100 g tuber, Table 4.2). Although the Gal contents of Kardal and UGE 51-16 1M CASS fractions are similar (31.7 vs. 35.0 mg/100 g tuber, Table 4.2), differences were found for Gal molar composition (8.6 mol% vs. 12.4 mol%, Table 4.1). The same accounts for the Xyl level of the 1M CASS fractions of Kardal and UGE 51-16.

The *4M CASS fractions* exhibited the lowest yields of all fractions, ranging between (3.9-6.6 g/100 g CWM). The 4M CASS fractions showed Glc as the main monosaccharide, and the potato varieties showed different Gal and Xyl contents for this fraction (Table 4.1 and 2). The UGE 45-1 had a low of Gal (6.2 mg/100 g tuber) and Xyl (5.8 mg/100 g tuber) content.

Glc represented between 93.5−94.3 mol% of sugars present in the *residues* (Table 4.1). The amount of Glc was slightly lower for UGE 45-1 residues (239.3 mg/ 100 g tuber) and UGE 51-16 residues (255.9 mg/ 100 g tuber) compared to wild-type (297.8 mg/ 100 g tuber).

Molecular weight distribution of the pectin fractions and their enzyme digests

Kardal (Figure 4.1A), UGE 45-1 (Figure 4.1B) and UGE 51-16 (Figure 4.1C) HBSS fractions both had M_w distributions ranging from ±1 to 3000 kDa. All HBSS fractions exhibited peaks at ±350 kDa, ±900 kDa and between 1 −10 kDa. The UGE transgenic lines had a high proportion of polysaccharides with a molecular mass between 1 − 10 kDa (Figures. 4.1B and 4.1C).
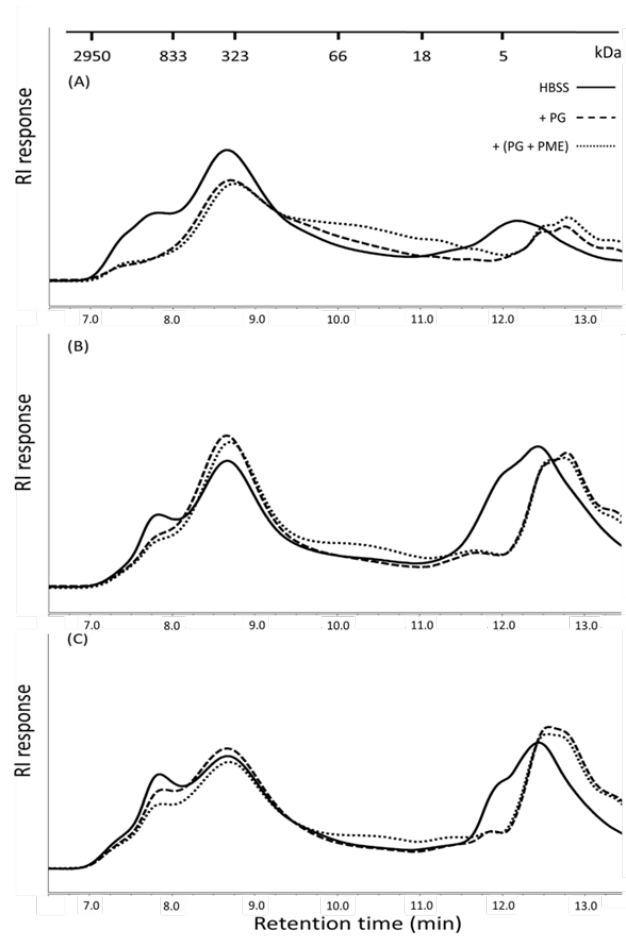
Figure 4.1. HPSEC profiles of the hot buffer-soluble solids (HBSS) fractions obtained from (A) Kardal, (B) UGE 45-1, and (C) UGE 51-16 following digestion with endopolygalacturonase (PG) or PG + pectin methylesterase (PME). Molecular masses of pullulan standards (in kDa) are indicated.

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Pectin backbone-degradation of HBSS fractions: Enzymatic degradation was performed to further elucidate the structural characteristics for HBSS, 1M CASS and 4M CASS fractions. The high M_w population of Kardal HBSS eluting within the time window of 7.0–10.5 min ($\pm 40 - 300$ kDa) decreased by 18 and 16% after PG and PG + PME incubation, respectively (Table 4.3). The corresponding high M_w population of UGE 45-1 HBSS (Figure 4.1B), showed a 1 and 3% decrease after digestion by PG and PG + PME, respectively (Table 4.3). The incubation of UGE 51-16 HBSS by PG resulted in a 4% reduction of high M_w population (Figure 4.1C; Table 4.3). The combination of PG + PME resulted in a 13% reduction of the high M_w population, quite similar to that of wild-type (Table 4.3).

Pectin side chain-degradation of HBSS fractions: Upon incubation with endoGal (Figure 4.2A), the high M_w population of Kardal HBSS decreased 58% (Table 4.3). For UGE 45-1 and UGE 51-16 (Figures 4.2B and 4.2C), these populations decreased by 66 and 74%, respectively (Table 4.3).

ExoGal incubation resulted in 20% degradation of the high M_w of Kardal HBSS fraction (Figure 4.2A; Table 4.3). Both UGE mutants showed a very limited degradation of the high M_w population by exoGal (Table 4.3).

Table 4.3. Degradation levels of high molecular weight pectin populations by endopolygalacturonase (PG), PG combined with pectin methylesterase (PME), β-endogalactanase (endoGal) and β-1,4-galactosidase (exoGal) of hot buffer-soluble solids (HBSS) fractions from Kardal, UGE45-1 and UGE51-16

Sample	Pectin backbone		Pectin side chains	
	PG	PG + PME	endoGal	exoGal
Kardal HBSS	18	16	58	20
UGE 45-1 HBSS	1	3	66	-[a]
UGE 51-16 HBSS	4	13	74	-[a]

Values were calculated from the peak areas of the high molecular weight HBSS fractions before and after enzymatic digestion within a time window of 7-10.5 min.
[a]No degradation.

Molecular weight distribution of the hemicellulose fractions and their enzyme digests

Two populations of hemicellulose (1M and 4M CASS) were investigated using enzymatic degradation by XEG alone and by XEG in combination with Celluclast by HPSEC.

The Kardal 1M fraction had a molecular mass distribution ranging from ± 1 to 400 kDa (Figure 4.3A) and contained two major populations with molecular mass at ± 5 and ± 80

kDa. Upon XGE incubation, the M_w distribution (±30 to 300 kDa) shifted slightly to lower M_w (Figure 4.3A). The combination of XEG and Celluclast lowered the M_w range to ±3 – 300 kDa (Figure 4.3A). The Kardal 4M hemicellulose fraction ranged from ±15 to 300 kDa with the main peak eluting at ±80 kDa (Figure 4.3B). The M_w distribution ranged from ±1 to ±30 kDa after XEG incubation (Figure 4.3B). The combination of XEG and Celluclast completely degraded the polysaccharides (Figure 4.3B). The 1M and 4M CASS fractions and their enzyme digests from UGE transgenic lines showed similar elution profiles as found for of Kardal (Supplementary data, Figures 4.1S and 4.2S, respectively).
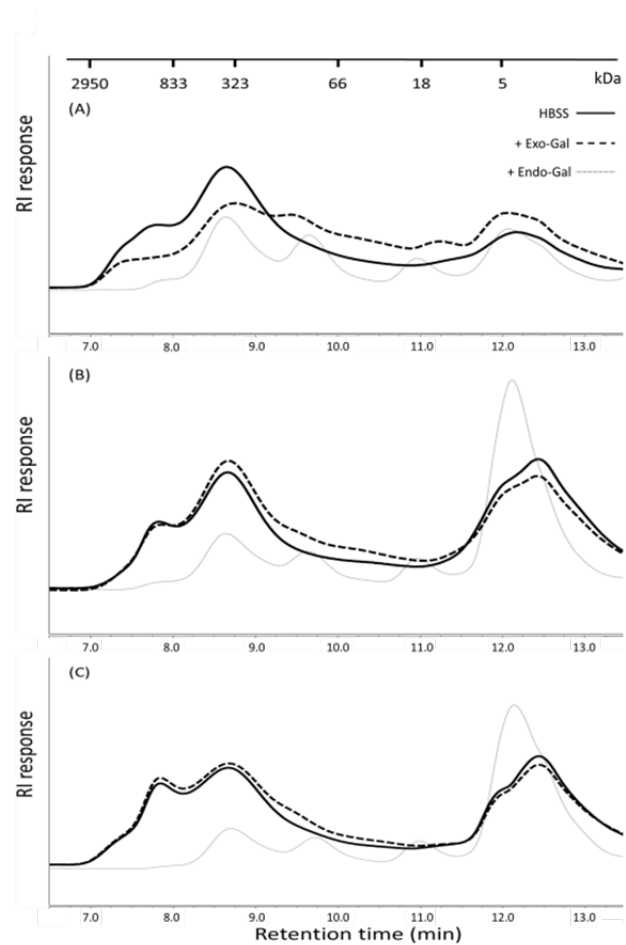
Figure 4.2. HPSEC profiles of the hot buffer-soluble solids (HBSS) fractions obtained from (A) Kardal, (B) UGE 45-1, and (C) UGE 51-16 following digestion with β-endogalactanase (endoGal) or β-galactosidase (exo-Gal). Molecular masses of pullulan standards (in kDa) are indicated.

The XEG + Celluclast digests were further analyzed by using MALDI-TOF MS (data not shown). XXG and XSG repeating units (RU) were the main degradation products of the combined action of XEG and Celluclast. We consider XXG to originate from XXGG by removal of a terminal glucose by the enzyme mix and similarly XSG and XSGG as representing the same RU. The ratios of (XSGG + XSG)/ (XXGG + XXG) were calculated based on the intensity of corresponding mass peaks. The peak ratios of RU XSGG to XXGG based on the peak intensities of (XSGG + XSG) and (XXGG + XXG) are shown in Table 4.4. The 1M CASS fractions of UGE 45-1 and UGE 51-16 exhibited higher (XSGG + XSG) / (XXGG + XXG) ratios than that of the wild-type (1.3 vs. 0.8, Table 4.4). The 4M CASS fraction of UGE 45-1 displayed the highest ratio of (XSGG + XSG)/ (XXGG + XXG) (1.6, Table 4.4). UGE 51-16 4M CASS had a lower (XSGG + XSG)/ (XXGG + XXG) ratio than the wild type (0.5 vs 1.2 of wild-type, Table 4.4).
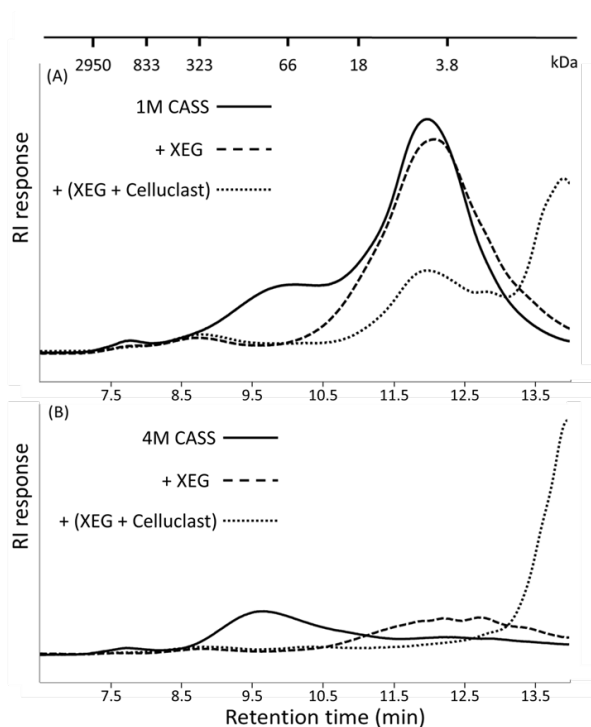
Figure 4.3. HPSEC profiles of (A) 1M and (B) 4M concentrated alkali soluble solids (CASS) fractions obtained from Kardal, following digestion with endo-β-1,4-glucanase (XEG) or XEG in combination with Celluclast. Molecular masses of pullulan standards (in kDa) are indicated.

Table 4.4. The intensities of peak ratios of repeating units XSGG to XXGG based on mass spectra of 1M and 4M concentrated alkali soluble solids (CASS) fractions obtained from Kardal, UGE 45-1, and UGE 51-16 after enzymatic degradation

Name	1M CASS	4M CASS
Kardal	0.8[a]	1.2
UGE 45-1	1.3	1.6
UGE 51-16	1.3	0.5

[a]1M and 4M CASS fractions were degraded with xyloglucan-specific endo-β-1,4-glucanase in combination with Celluclast and digests were analyzed by MALDI-TOF MS for the XSGG : XXGG ratio.

Discussion

Previous research on CWM from UGE transgenic lines was hampered by a high content of starch (50% w/w) present in the isolated CWM (Oomen et al., 2004). Furthermore, Oomen et al. (2004) only discussed the monosaccharide content of the CWM (w/w %), and thus the overall transgenic modifications made within the UGE transgenic lines remained unidentified. In the present study, more than 99.5% of the starch was removed from the potato varieties by using the described procedure (Huang et al., 2016). Obtaining CWM with relatively low starch levels (0.28 −0.31 g/100 g tuber) is essential of characterization of cell wall polysaccharides present. First, the targeted structure, galactan side chains, of transgenic modification will be discussed. Then, the side effects of UGE transgenic modification will be addressed.

Galactan side chains (targeted structures for modification)

The Gal content (mg/ 100 g tuber) of UGE 45-1 CWM was, as expected, higher (38%) than that of the Kardal following the expression of the gene encoding UGE enzymes (Table 4.2). Conversely, the Gal content (mg/ 100 g tuber) of UGE 51-16 CWM was unexpectedly 17% lower than that of Kardal (Table 4.2). The latter may be due to the reversible conversion reaction of UDP-Gal into UDP-Glc (Atmodjo et al., 2013) that changes the corresponding UDP-Gal and UDP-Glc levels available for cell wall polysaccharides synthesis. Both UGE 45- and UGE 51- genes encoded the UGE enzyme, but belonged to different clusters as reported previously (Oomen et al., 2004). The comparison of amino acid sequence of UGE45- and UGE51- gene pointed to a 67% identity (Supplementary Data, Figure 4.3S), which may explain the different the effects on Gal content after introduction of UGE 45- or UGE 51- genes.

The Gal:Rha ratio (Figure 4.4A) reveals an indication of the lengths of the pectin side chains according to the molar sugar composition. The CWM pectin of UGE 45-1 had longer galactan side chains than the wild-type (Figure 4.4A), e.g., the Gal:Rha ratios of UGE 45-1 and Kardal CWM are 59 and 34, respectively. The same accounts for the HBSS and DASS fractions of UGE 45-1 and Kardal (plus 33% and 40%, respectively). The ChSS

fraction of UGE 45-1 had a quite similar side chain length than that of wild-type (Gal:Rha = 47 vs. 41 of wild type). All UGE 45-1 pectin fractions contained higher Gal levels than that of wild-type, indicating that the observation of longer galactan side chains present in the CWM is valid for all different pectin populations investigated. Although the UGE 51-16 CWM indicated the presence of shorter side chains (Gal:Rha = 25 vs. 34 for wild-type), this effect was only found for the UGE 51-16 ChSS fraction (Gal:Rha = 23 vs 41 for wild-type, Figure 4.4A). The HBSS and DASS fractions of UGE 51-16 maintained the same length of side chains in comparison to that of wild-type (Figure 4.4A). The UGE gene encoding pectin synthesis enzymes affects each pectin population individually, resulting in rather different pectin populations.
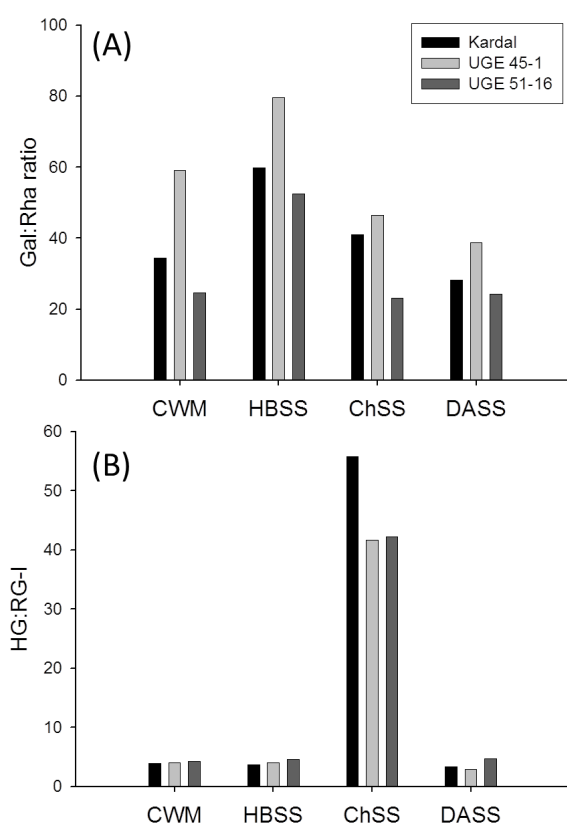
Figure 4.4. Characteristics of pectin present in cell wall material (CWM), hot buffer-soluble solids (HBSS), chelating agent-soluble solids (ChSS), and diluted alkali-soluble solids (DASS) fractions from Kardal (wild-type), UGE 45-1 and UGE 51-16. (A) Gal:Rha ratio and (B) HG:RG-I ratio based on the molar sugar composition.

The enzymatic degradation of the various fractions by endoGal shows that the pectin side chains of UGE transgenic lines are rich in (1→4)-linked galactan. The incomplete degradation by endoGal of UGE 45-1 HBSS fractions may be caused by the hindrance of endoGal activity by galactan side chain substitution. This suggestion is strengthened by the limited degradation of UGE 45-1 and UGE 51-16 HBSS by exoGal compared to wild-type (Figure 4.2, Table 4.3). The exoGal used is an exo-acting enzyme that degrade β-(1→4)-linked galactan, exclusively releasing single Gal (Van de Vis, 1994). Based on exoGal degradation (Figures 4.2 and 4.3), a more complex substitution of the galactan side chains may present in both UGE transgenic lines. Such altered side chains structure may result from adaptation of the synthesis machinery to maintain the cell wall strength.

Side effects of transgenic modification

Pectin backbone: Figure 4.4B shows the HG:RG-I ratios of CWM and its fractions. The pectin backbone of UGE 45-1 and UGE 51-16 remained similar to that of the wild-type in all fractions expect for calcium-bound pectin (ChSS fractions, high HG:RG-I ratio, Figure 4.4B). The HG:RG-I ratios of UGE 45-1 and UGE 51-16 ChSS fractions showed a decrease of 25% and 24% compared to that of wild-type (HG:RG-I = 55.8:1), respectively. Furthermore, UGE transgenic lines had a higher methyl-esterification and lower acetylation of the cell wall pectin than Kardal. UGE 45-1 ChSS had a higher methyl-esterified pectin (DM = 55 vs DM = 36 of wild type, Table 4.1), which provided a lower net charge of the pectin. The pectins might be bound to other polysaccharides by (non)covalent crosslinking to form the cell wall architecture (Cosgrove, 2005). The side effect on pectin backbone might provide suitable strength for cell wall structure upon modification of the pectin side chains. Pectin esterification has been found to occur as a side effect in RGL and β-Gal transgenic lines (Huang et al., 2016), but has been reported for the first time in the UGE transgenic lines. Based on the present research, the UGE transgenic modification showed side effects on pectin esterification as well.

Xyloglucan: Although the 1M and 4M CASS fractions of the UGE transgenic lines had similar constituent monosaccharide compositions as those from the wild-type (Tables 4.1 and 4.2), the XXGG and XSGG xyloglucan RU were predominately in wild-type and UGE transgenic lines but exhibited different proportions (Table 4.4). Vincken et al. (1996) reported that the XXGG-type xyloglucan are predominant in potato xyloglucan, which was also found in the UGE transgenic lines and its background Kardal. Different proportions of XSGG/XXGG RU in the 1M and 4M fractions of all potato varieties indicated that the side effects on xyloglucan also altered the xyloglucan population. The changes in the xyloglucan structure might occur during the construction of the cell wall network that occurs during plant growth (Cosgrove, 2005). Since the side chains of xyloglucan has been reported to fold around the xyloglucan backbone (Umemura & Yuguchi, 2005), such a folding formed by different side chains structures may provide different interactions between xyloglucan

and cellulose (Zhang et al., 2011). All the pectin or xyloglucan populations showed different targeted modification or side effects. The potato transgenic lines need to construct a suitable cell wall architecture to maintain the strength of cell wall after the targeted modification of galactan side chains. Therefore, the pectin and xyloglucan structures may have been biosynthetically modified individually.

Cellulose network: The extracted residues mainly consisted of cellulose. The trace contents of other monosaccharides than Glc detected might originate from pectin or xyloglucan strongly bond to cellulose (Levy et al., 1997; Zykwinska et al., 2005). Both the amount of total carbohydrate of CWM (mg/100 g tuber) and the Glc content (mg/100 g tuber) from UGE 45-1 residue showed a decrease of 19% and 20% compared to wild-type, indicating at the relative amount of cellulose was not affected in UGE 45-1. The same accounts for UGE 51-16 as it showed a ±14% reduction of both total carbohydrates and Glc.

Mechanism of non-targeted alteration of cell wall polysaccharides: The alteration of the non-targeted monosaccharides in the cell wall polysaccharides from UGE transgenic lines was probably caused by the changed of UDP-sugar interconversion machinery. The complete UDP-sugar interconversion pathway might be influenced by the activity of the UGE enzyme. Both UGE 45- and UGE 51- genes encoded UGE enzyme, but resulted in a different modification of the galactan chains as well as other polysaccharides. The complete enzyme system might have been altered and thereby influenced the levels of individual UDP-sugar, but more evidence is needed as evidence. These UDP-sugars might consequently affect the monosaccharides in the cell wall during plant growth.

Conclusions

UGE 45-1 exhibited higher levels of Gal and longer side chains in all pectin fractions, compared to those found for the wild-type. Conversely, UGE 51-16 pectin polysaccharide fractions possessed similar or shorter side chain lengths than the wild-type. In addition, non-targeted structures had been changed in each pectin or xyloglucan polysaccharide population of UGE transgenic lines investigated. The results show that introducing the gene encoding UGE enzyme can have multiple effects that were not analyzed in previous studies.

Acknowledgements

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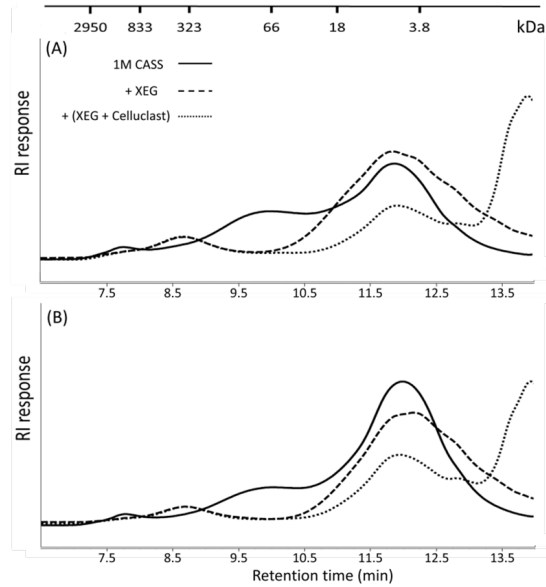
Figure 4.1S. HPSEC profiles of 1M concentrated alkali soluble solids (CASS) fractions obtained from (A) UGE 45-1 and (B) UGE 51-16 following digestion with endo-β-1,4-glucanase (XEG) or XEG in combination with Celluclast. Molecular masses of pullulan standards (in kDa) are indicated.
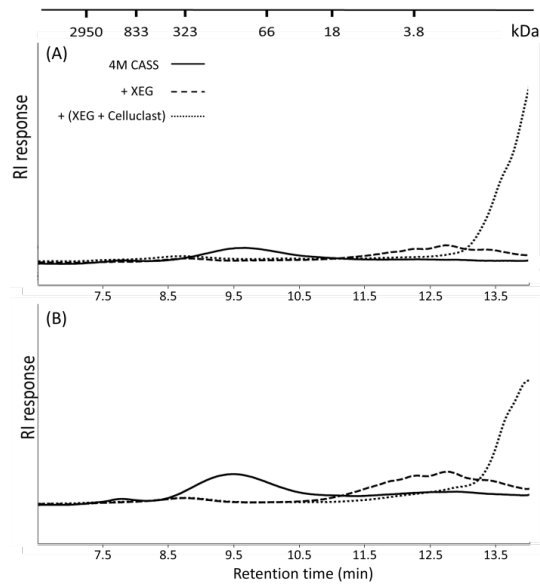
Figure 4.2S. HPSEC profiles of 4M concentrated alkali soluble solids (CASS) fractions obtained from (A) UGE 45-1 and (B) UGE 51-16 following digestion with endo-β-1,4-glucanase (XEG) or XEG in combination with Celluclast. Molecular masses of pullulan standards (in kDa) are indicated.

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Query   2    GEM----SKSILVTGGAGYIGSHTVLQLLLGGYKTVVIDSLDNSSEIAVKRVKEIAG-EY   56
             GEM      ++ILVTGGAG+IG+HTV+QLL  G+K  +ID+  NS E AV RV+E+ G +
Sbjct   2    GEMGVQCQENILVTGGAGFIGTHTVVQLLNEGFKVTIIDNFHNSVEEAVDRVRELVGPQL   61


Query   57   GSNLSFHKVDLRDKPAVEEIFRSNKFDAVIHFAGLKAVGESVEKPLMYYDNNLIGTITLL  116
               NL FH  D+R+K  +E++F    +F AV+HFAGLKAVGESV +P +Y++NNLIG+ITL
Sbjct   62   SQNLEFHLGDIRNKDDLEKLFSKKEFAAVVHFAGLKAVGESVVQPFLYFENNLIGSITLY  121


Query   117  EIMAAHGCKRLVFSSSATVYGWPKVVPCTEEFPLSAANPYGRTKLFIEEICRDVQNADSE  176
             +MA + CK+LVFSSSATVYG P+ VPC E+F L A NPYGRTKLF+E+I RD+Q AD E
Sbjct   122  SVMAKYNCKKLVFSSSATVYGQPEKVPCVEDFELKAMNPYGRTKLFLEDIARDIQKADQE  181


Query   177  WKIILLRYFNPVGAHPSGRIGEDPRGIPNNLMPFVQQVAVGRRKELTVYGTDYGTKDGTG  236
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Sbjct   182  WNIILLRYFNPVGAHESGKLGEDPKGIPNNLMPYIQQVAVGRLPELNVYGNDYPTPDGTA  241


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Query   296  MSGRRPGDAEIVYAATEKAERELKWKAKYGIEEMCRDQWNWAKKNPYGY    344
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Sbjct   302  MCPRRPGDATAVYASTEKAEKELGWKAKYGINEMCRDQWKWASQNPWGY    350
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Figure 4.3S: The comparison of amino acid sequence of UGE 45- and UGE 51- genes.

Chapter 5

Extended evaluation of cell wall polysaccharide composition in transgenic potatoes

Abstract

Pectin is an integral component of primary cell walls and contributes to their mechanical strength. In this study, we analyze four wild-type potato varieties and 31 transgenic lines to determine the transgene effects on targeted structures, including rhamnogalacturonan I (RG-I) and homogalacturonan (HG), neutral side chains (galactan / arabinangalactan), acetylation of pectin, and cellulose level. In addition to the targeted modification, associated side effects in transgenic lines resulted in changes in non-targeted polymers, in the acetylation and methyl-esterification of pectin, and in pectin and (hemi)cellulose contents. The modification from the pectin backbone or pectin side chains transgenic lines either increased or decreased simultaneously the HG:RG-I ratio, side chains length and methyl-esterification of pectin. The pectin esterification transgenic line exhibited only limited side effects. The cellulose level targeting transgenic lines had an unexpected high HG:RG-I ratio and longer pectic side chains when compared to the wild-type. The pectin content remained at the same level after the cellulose modification as found for the wild-type. The results clearly show that it is necessary to evaluate not only the effects on the modification of targeted structures but also on the complete cell wall composition.

Introduction

Potato tubers are among the most important food crops for human consumption (Camire et al., 2009). Most potato cultivars are tetraploid, providing a huge genetic diversity for breeding purposes (Lehesranta et al., 2005) The diverse genetic background presents a challenge for transgenic modification of potato and can cause a difficulty for selecting appropriate transgenic events in same cases (Jansky, 2009). The detailed analysis of different cell wall polysaccharide populations from wild-type potato and transgenic lines can provide information on the effects of transgenes on the entire cell wall. This procedure is time-consuming, allowing the detailed analysis of only a small number of samples, thus, a new strategy for evaluating modified cell wall polysaccharides might be useful.

Potato pectin is composed of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). HG is composed of a linear backbone of consecutive 1,4-linked-α-d-galacturonic acid (GalA) units, with the GalA residues being partly methyl-esterified at C-6. The backbone of RG-I consists of 4-α-d-GalpA-(1,2)-α-l-rhamnose (Rha) disaccharide as repeating units, and the side chains are composed of α-l-arabinofuranosyl and/or β-d-galactopyranosyl residues (Albersheim et al., 1996).

Plant cell wall materials (CWM) can be modified by the expression of hydrolytic enzymes *in vivo* to modify targeted structures (Øbro et al., 2010). The modification of plant cell walls *in planta* has mainly been monitored by microscopy combined with antibody immunolabeling (Knox et al., 1990; Jones et al., 1997; Willats et al., 1998) and Fourier transform infrared (FTIR) spectroscopy (Chen et al., 1998). Obtained microscopy spectra, in combination with molar sugar compositions, can provide qualitative evidence of modified CWM present in transgenic lines (Oomen et al., 2004a). Although Moller et al. (2007) described a high-throughput microarray for screening cell wall polymers, antibodies for immunolabeling are not readily available and are expensive. Additionally, this method can only be used semi-quantitatively. The transgenic effects on potato tubers or other non-modified cell wall polysaccharides have not always been sufficiently studied (Sørensen et al., 2000; Oomen et al., 2004a; Martín et al., 2005; Øbro et al., 2009), since these studies only focused on the targeted structures. Therefore, the entire range of effects on other cell wall polysaccharides has been poorly studied, hampering the complete evaluation of potato transgenic lines.

Pectin transgenic lines exhibit specific effects. The side chain modification (β-galactosidase [β-Gal]) transgenic lines exhibited shortened galactan side chains, but were also found to have distinct alterations in the pectin backbone, pectin esterification and xyloglucan structure (Chapters 2 & 3). Pectin backbone-modification in rhamnogalacturonan lyase (RGL) transgenic lines resulted in side effects altering pectin esterification (Huang et al., 2016). While a very detailed view can be useful, time and

sufficient amounts of material from potato transgenic lines may not be available for the initial screening stage. Therefore, a brief but careful examination including cell wall yield, sugar composition, and pectin characteristics like HG:RG-I or galactose(Gal):Rha ratio can provide an improved screening output and thereby provide detailed information on modified cell wall polysaccharides.

In this study, we evaluated wild-type and transgenic lines regarding targeted structures as well as non-targeted structures. The proposed strategy was applied to novel potato transgenic lines with transgenes expressing galacturonosyl (Gaut1) and UDP-rhamnose synthase (RhaS) enzymes. Other available transgenic lines reported previously were re-examined by the proposed evaluation method to provide lacking information on non-targeted polymers.

Materials and Methods

Transformation of potato cultivars and transgenic lines

Based on the targeted modification site, the potato transgenic lines were divided into four categories: (A) pectin backbone, (B) pectin side chains, (C) pectin esterification and (D) cellulose level transgenic lines.

The following transgenic lines and respective wild-type potato (Table 5.1) were analyzed: (1) Desiree and derived transgenic lines expressing galacturonosyl transferase1 (Gaut1) (Atmodjo et al., 2011) or UDP-rhamnose synthase (RhaS) (Reiter & Vanzin, 2001); (2) Karnico and transgenic lines expressing RGL (Oomen et al., 2002; Huang et al., 2016) or β-Gal (Martín et al., 2005; Huang et al., 2016); (3) Posmo and transgenic lines expressing endo-1,4-β-d-galactanase (endoGAL) (Sørensen et al., 2000), endo-α-1,5-l-arabinase (eARA) (Skjøt et al., 2002), both endo-α-1,5-L-arabinase and endo-1,4-β-D-galactanase (double construct: endoGAL + eARA) (Øbro et al., 2009; Cankar et al., 2014), or pectin acetylesterase (PAE) (Orfila et al., 2012); and (4) Kardal and derived transgenic lines expressing sense-cellulose synthase (CesA) (Oomen et al., 2004b), anti-sense cellulose synthase (asCesA) (Oomen et al., 2004b), class-specific regions of cellulose synthase (CSR) (Oomen et al., 2004b), UDP-glucose (Glc) 4-epimerase (UGE) (Oomen et al., 2004a), or a double construct (eGAL + eARA) (Cankar et al., 2014). Gene expression and the corresponding observations on the polymer-modifying enzyme activities are described in Table 5.1.

Since the Gaut1 and RhaS transgenic lines represent the first introduction of those enzymes into the potato genome, the cloning procedure is briefly described below. The procedure of other transgenic lines can be found in the references list of Table 5.1. The double construct (endoGAL + eARA) has been previously studied in the Posmo background (Cankar et al., 2014). In this research, both genes were also introduced into the

Kardal background. The other potato transgenic lines have been studied before. The available information of potato transgenic lines observed from previous research is summarized in Table 5.1.

Cloning of the constructs overexpressing rhamnosesynthase (RhaS) and galacturonosyltransferase (Gaut1)

To over-express the *rhamnosesynthase* gene or the *galacturonosyltransferase1* gene in potato the Gateway® cloning system was used. The coding sequences of the genes were cloned from cDNA of tuber from potato cultivar Kardal and put under the control of the cauliflower mosaic virus (35S) promoter (Odell et al., 1985). Sequencing primers were designed to cover the entire length of the coding region in both directions. Two independent clones were sequenced to confirm the fidelity of amplification with *Pfu* polymerase. The final constructs were transferred to *Agrobacterium tumefaciens* strain LBA4404 for plant transformation.

Transformation of RhaS and Gaut1 transgenic lines

Transformation of the binary constructs was carried out as described by Visser (1991). Potato cultivar Desiree was used for transformation experiments. Potato stem segments were co-cultivated for 3 days with *Agrobacterium tumefaciens* carrying plasmid RhaS or Gaut1. After 4-8 weeks, regenerating shoots were collected and cultured on MS (Murashige & Skoog, 1962) medium including 3% sucrose supplied with 50 mg/L kanamycin.

Transgenic targeted polymers and side effects

A summary of the potato transgenic lines based on modification of (A) pectin backbone, (B) pectin side chains, (C) pectin esterification and (D) cellulose level and their possible side effects —pectin backbone (HG and RG-I), pectin side chains (galactan or arabinogalactan side chains), acetylation (DA) and methyl-esterification (DM) of cell wall pectin, pectin content (arabinose [Ara], Rha, Gal plus GalA), (hemi)cellulose content (xylose [Xyl], mannose [Man] plus Glc) and non-targeted monosaccharide—, is presented in Table 5.2. Along with the targeted modifications marked by 'X', the other (empty) cells represent potential side effects (Table 5.2).

Potato tuber preparation

Potato tubers were grown in a greenhouse (Unifarm, Wageningen UR, Wageningen, The Netherlands) and were harvested in 2011–2013. The tubers were peeled and diced into 5 mm cubes, which were then rapidly frozen in liquid nitrogen to preserve enzymatic activity. The samples were stored at −20 °C until use. The frozen potato samples were freeze-dried and homogenized prior to analysis.

Table 5.1. Wild-type potato varieties (Karnico, Posmo, Kardal and Desiree), their transgenic lines targeting (A) pectin backbone (B) pectin side chains; (C) pectin esterification and (D) cellulose levels and reported changes in cell wall polysaccharides.

Category	Transgenes (symbol)[a]	Wild-type	Lines	Observed modification to structures	Information available			References
					CWM	Fresh weight	Pectin characterization	
A	UDP-rhamnose synthase (RhaS)	Desiree	1, 2, 3	Increase RG-I element in pectin backbone[b]	No	No	No	Not available[c]
	Galacturonosyl transferase1 (Gaut1)	Desiree	6, 16, 18, 21, 23, 26	Increase HG element in pectin backbone[b]	No	No	No	Not available[c]
	Rhamnogalacturon lyase (RGL)	Karnico	9, 18	Shorter RG-I element in pectin backbone	Yes	Yes[d]	Yes[d]	Oomen et al. (2002)
B	β-galactosidase (β-Gal)	Karnico	7, 14, 19, 27	Shorter galactan side chains of RG-I	Yes	Yes[d]	Yes[d]	Martin et al. (2005)
	Endo-1,4-β-D-galactanase (endoGAL)	Posmo	13.1	Shorter galactan side chains of RG-I	Yes	Yes	No	Sørensen et al. (2000)
	Endo-α-1,5-L-arabinase (eARA)	Posmo	7.2	Shorter arabinan side chains of RG-I	Yes	No	No	Skjøt et al. (2002)
	The double construct (endoGAL+eARA)	Posmo	10, 13	Shorter galactan and arabinan side chains of RG-I	Yes	No	Yes	Cankar et al. (2014) Øbro et al. (2009)
	The double construct (endoGAL+eARA)	Kardal	25, 26, 27	Shorter galactan and arabinan side chains of RG-I	No	No	No	Not available[c]
	UDP-Glc 4-epimerase (UGE)	Kardal	45-1, 51-15, 51-16, 51-19	Elongate galactan side chains of RG-I	Yes	Yes[e]	Yes[e]	Oomen et al. (2004b)
C	Pectin acetyl esterase (PAE)	Posmo	31	Lower acetyl substrate of HG	Yes	No	Yes	Orfila et al. (2012)
D	Sense-cellulose synthase (CesA)	Kardal	39	Increased cellulose level	Yes	No	No	Oomen et al. (2004a)
	Anti-sense cellulose synthase (asCesA)	Kardal	47	Decreased cellulose level	Yes	No	No	(Oomen et al. (2004a)
	Class-specific regions of cellulose synthase (CSR)	Kardal	4-8, 2-1	Decreased cellulose level	Yes	No	No	(Oomen et al. (2004a)

Abbreviations: homogalacturonan (HG); rhamnogalacturonan I (RG-I).
[a]Transgenic nomenclature: symbol + line, i.e., Gaut1, line 16, is labeled Gaut1-16 in this study.
[b]Data is available only for Arabidopsis. RhaS: Reiter and Vanzin (2001); Gaut1 : Atmodjo et al. (2011).
[c]New transgenic lines in potato.
[d]From this thesis: Huang et al., (2016).
[e]From this thesis: Chapter 4.

Isolation of CWM from potato tubers

To promote starch gelatinization and inactivate endogenous enzymes, 5 g of the dry material was added to 25 mL of sodium acetate buffer (pH 5.2) and incubated at 80 °C for 30 min. After two rounds of starch gelatinization and enzymatic degradation, the cell wall polysaccharides were precipitated by adding ethanol to a final concentration of 70% (v/v). The detailed description has been reported previously (Huang et al., 2016). The washed pellet was lyophilised, yielding the CWM.

Determination of constituent monosaccharide compositions

The CWM and fractions obtained were analyzed to determine the constituent monosaccharide composition using a prehydrolysis step with 72% (w/w) sulfuric acid at 30°C for 1 h. Subsequently, the samples were hydrolyzed with 1 M sulfuric acid at 100°C for 3 h and sugars released were analysed as their volatile alditol acetates by GLC, as described previously (Huang et al., 2016). The total uronic acid content was determined using an automated-m-hydroxydiphenyl assay (Thibault, 1979). The total uronic acid content measured was attributed to GalA. The starch content in CWM was analyzed enzymatically using the Megazyme Total Starch Kit (Megazyme, Wicklow, Ireland). Starch derived glucose (Glc) was subtracted from total Glc to obtain cell wall Glc.

The molar ratios of galactose:rhamnose (Gal:Rha) or (Ara + Gal):Rha can be used to determine the lengths of galactan or arabinogalactan side chains, respectively. Because the pectin backbone is composed of HG (100% GalA) and RG-I (Rha:GalA ratio of 1:1), the HG:RG-I molar ratio can be calculated as the molar ratio of (GalA - Rha): (2 × Rha).

Degrees of methyl-esterification (DM) and acetylation (DA)

Methyl esters and acetyl groups present on the polysaccharides were released by saponification and measured by high-performance liquid chromatography (Voragen et al., 1986). The degree of acetylation and methyl-esterification are calculated as mole methyl esters or acetyl groups per 100 mole GalA. One mole GalA can carry only one mole methyl esters and two moles acetyl groups.

Strategy for evaluating CWM in transgenic potato

The strategy used in this study is shown in Figure 5.1. First, CWMs were isolated from wild-type potatoes and their transgenic lines (Figure 5.1A). Second, the constituent monosaccharide composition and pectin esterification of CWMs were analyzed (Figure 5.1B). Based on the molar monosaccharide compositions, the side chain length was calculated using Gal:Rha and (Ara + Gal):Rha ratios (Figure 5.1C). The HG:RG-I ratio provides structural information about the pectin backbone (Figure 5.1C). The monosaccharide compositions were then used to determine the monosaccharide yield from

fresh potato tubers in order to understand the effect on the complete tuber (Figure 5.1C). The acetylation and methyl-esterification (DA and DM) of pectin were included in the analysis (Figure 5.1C). The pectin (sum of Ara, Rha, Gal and GalA) and (hemi)cellulose (sum of Xyl, Man and Glc) contents in the fresh weight potato basis (mg/100 g tuber) provided the proportions of polysaccharides after transgenic modifications of cell wall polysaccharides (Figure 5.1C).

The effect of the modifications in transgenic potato transgenic lines was measured against the corresponding values of the wild-type (Figure 5.1D). The potato transgenic lines were evaluated for effects on both the specifically targeted structures as well as for possible side effects (Figure 5.1E).
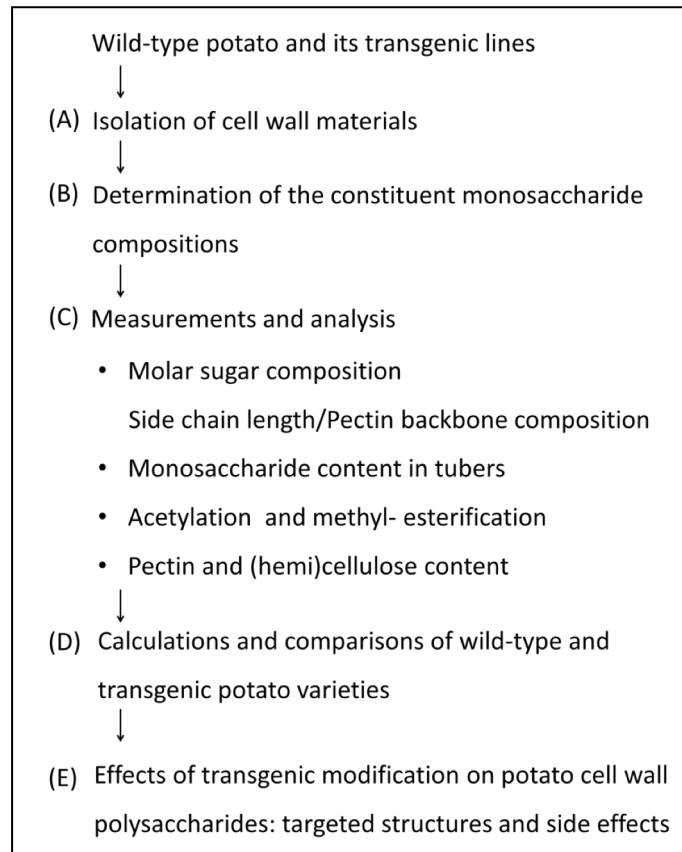
Figure 5.1. Flow chart for screening and evaluating potato genotypes

Results and Discussion

In the present study, transgenic lines were categorized based on modifications of (A) the pectin backbone, (B) pectin side chains, (C) pectin esterification and (D) cellulose level (Table 5.1). According to our strategy, in addition to analyzing the modification of targeted structures, we also considered side effects (Table 5.2). The detailed information including molar composition, individual monosaccharide content (mg/ 100 g tuber) present in CWM, acetylation (DA) and methyl-esterification (DM) of cell wall pectin for all wild-type and transgenic lines are available in Supplementary Tables 5.1S and 5.2S.

Most potato CWMs were found to be rich in Glc and Gal. In contract, the endoGAL and endoGAL + eARA transgenic lines showed low molar proportions of Gal. Minor quantities of Rha, Ara, Xyl and Man were present in the CWMs of all varieties. Following the evaluation strategy as presented in Figure 5.1 and to simplify the comparison, we only presented deviations found for the transgenic lines from the values found for the wild-type potato. Each category of transgenic modification will be discussed separately.

Modification of the pectin backbone

The gene encoding for the RhaS enzyme catalyzes the conversion of UDP-Glc to UDP-Rha (Reiter & Vanzin, 2001). The UDP-Rha has been correlated to the amount of Rha in the cell wall during plant biosynthesis of RG-I element in the pectin backbone in Arabidopsis tissue (Reiter & Vanzin, 2001). Gaut1 is involved in synthesizing HG by inserting GalA into pectin HG element in Arabidopsis (Atmodjo et al., 2011). Introduction of the gene encoding for RGL, which acts on the RG-I backbone into potato (Oomen et al., 2002), resulted in the release of galactan-rich RG-I elements (Huang et al., 2016).

Targeted structure: In Figure 5.2, the bars marked with an asterisk represent the targeted structure modification. The RhaS-1 and RhaS-3 transgenic lines showed a reduced Rha content in the CWM (minus 32% and 41%, respectively), whereas RhaS-2 showed an increased Rha content (plus 29%, Figure 5.2). So far, the RhaS gene has been introduced only into the genome of Arabidopsis and only differences of less than 5% in the molar sugar composition of Rha have been shown while no information on the amounts of monosaccharides in the CWM were provided (Diet et al., 2006). The Rha levels in CWM influenced the RG-I elements present in the pectin backbone. Despite the variable effect of the lines on the Rha content, the HG:RG-I ratios from all RhaS transgenic lines were approximately 50% higher than those in the wild-type (Figure 5.3A), indicating relatively more HG structure elements than RG-I segments for all three transgenic lines.

Table 5.2. Categories of potato transgenic lines based on modification of (A) the pectin backbone, (B) pectin side chains, (C) pectin esterification, or (D) cellulose levels and their cell wall polysaccharide structures expected to be affected.

Category	Transgenes encoding enzymes	Pectin backbone		Pectin side chains		Pectin esterification		Pectin content	(hemi)Cellulose content	Non-targeted monosaccharide
		HG	RG-I	Galactan	Arabinogalactan	DA	DM			
A	UDP-rhamnose (RhaS)		X					X		
	Galacturonosyl transferase1 (Gaut1)	X						X		
	Rhamnogalacturon lyase (RGL)		X					X		
B	β-galactosidase (β-Gal)			X				X		
	Endo-1,4-β-D-galactanase (endoGAL)			X				X		
	Endo-α-1,5-L- arabinase (eARA)				X			X		
	The double construct (endoGAL+eARA)			X	X			X		
	UDP-Glc 4-epimerase (UGE)			X				X		
C	Pectin acetyl esterase (PAE)					X				
D	Sense-cellulose synthase (CesA)								X	
	Anti-sense cellulose synthase (asCesA)								X	
	Class-specific regions of cellulose synthase (CSR)								X	

X indicates the expected modification of each transgenic line. The other cells without indication were the possible side effects.
Abbreviations: homogalacturonan (HG), rhamnogalacturonan I (RG-I), pectin esterification including acetylation (DA) and methyl-esterification (DM).

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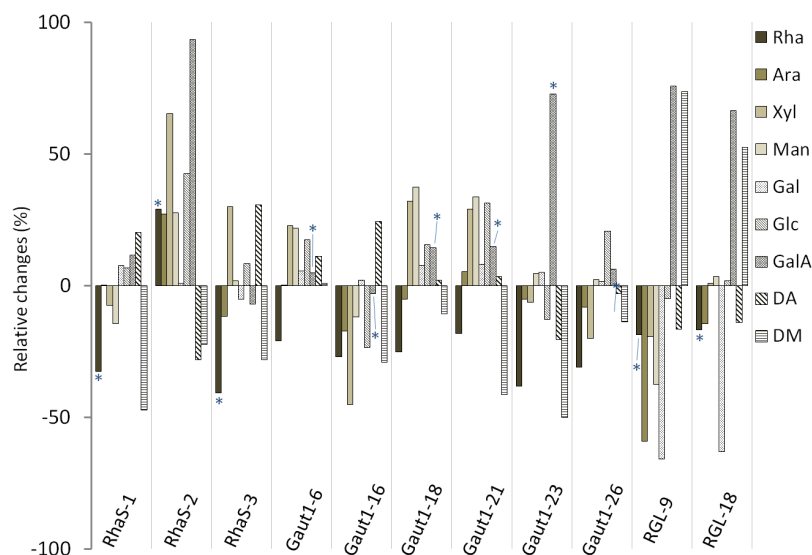
Figure 5.2. Effect of transgenic modification of pectin backbone in RhaS (1, 2, and 3), Gaut1 (6, 16, 18, 21, 23, and 26) and RGL (9 and 18) transgenic lines on monosaccharide contents, acetylation (DA) and methyl-esterification (DM) of cell wall pectin. The changes of monosaccharide contents (mg/100 g tuber), DA and DM are relative to corresponding values of the wild-type potato variety. The asterisk denotes the compound which was affected due to the targeted modification.

The Gaut1 transgenic lines showed an increase in GalA content (mg/100g tuber) in the CWM ranging from 5%–15%, except for Gaut1-23 (plus 73%) and Gaut1-16 (minus 7%, Figure 5.2). The higher ratios of HG:RG-I present in the Gaut1 transgenic lines (Figure 5.3A) is not only caused by a higher GalA content but also by a lower Rha content (Figure 5.2), which indicates a reduction of RG-I elements in the pectin backbone. Previous studies indicated that Gaut1 catalyzes the transfer of GalA from UDP-GalA to HG (Sterling et al., 2006; Atmodjo et al., 2011), but information is missing on the total GalA content in the plant tissue making it impossible to judge the effects of Gaut1 enzyme on cell walls in their case.

The RGL transgenic lines showed reduced Rha contents in CWM (Figure 5.2), possibly due to cleavage of RG-I elements (shown by a high HG:RG-I ratio, Figure 5.3A). The reduction of Ara and Gal content in CWM (Figure 5.2) resulted in low (Ara+Gal):Rha or Gal:Rha ratios when compared to wild-type (Figure 5.3A). The release of neutral side chains-rich RG-I in RGL transgenic lines has been proven by the detailed characterization of isolated pectin fractions (Huang et al., 2016). Although the fractionation and characterization of pectin polysaccharides can provide clear advantages to recognize all

modifications in individual pectin fractions, our proposed screening strategy is more efficient when evaluating multiple transgenic lines.

Non-targeted changes: Also non-targeted structures were addressed to examine the entire effect of the transgenic modification of potato tubers. In Figure 5.2, the bars without an asterisk represent non-targeted monosaccharides and also changes in the pectin esterification (DA/DM) are considered as side effects. RhaS-1 and -3 displayed long galactan (Gal:Rha ratios: 33 vs. 21 for the wild-type) or arabinogalactan ([Gal+Ara]:Rha ratios: 36 vs. 23 for the wild-type) side chains (Figure 5.3A). RhaS-2 showed shorter side chains of galactan (Gal:Rha ratio: 18) or arabinogalactan ([Gal+Ara]:Rha ratio: 16) than the wild-type. When calculating the deviations with the Gal:Rha ratio found for the wild-type, the transgenic lines show approximate 60% increase in the ratio for RhaS-1 and RhaS-3 and a 22% decrease for RhaS-2, respectively (Figure 5.3A). The average increase in chain length for RhaS-1 and -2 but not for RhaS-3 matches nicely with the deviations found in Rha content.

Positive deviations of Gal:Rha and (Ara+Gal):Rha were found for all Gaut1 transgenic lines, ranging from 35% to 70%, while the increase of average chain length could easily be correlated to the reduced Rha levels (Figure 5.2). Furthermore, significant changes of DA in both positive and negative direction (Figure 5.2) were found for all transgenic lines, although not correlated to any increase or decrease of GalA. All RhaS and Gaut1 transgenic lines have lower methyl-esterification of cell wall pectin (Figure 5.2). The mechanisms of such side effects remain unclear.

Another way of judging the modification of cell wall polysaccharides is to calculate the pectin content (Ara, Rha, Gal plus GalA) and (hemi)cellulose content (Xyl, Man plus Glc) basis on the fresh weight of potato (mg/100 g tuber). The pectin and (hemi)cellulose contents in comparison to the corresponding values of wild-type can provide additional information for understanding the effects of transgenic modification of the entire cell wall (Figure 5.4A). RhaS and Gaut1 transgenic lines had higher (hemi)cellulose contents, excepted for Gaut1-16 and Gaut1-26 (Figure 5.4A). RGL transgenic lines resulted in lower pectin contents but had limited effect on (hemi)cellulose contents (<10%, Figure 5.4A).
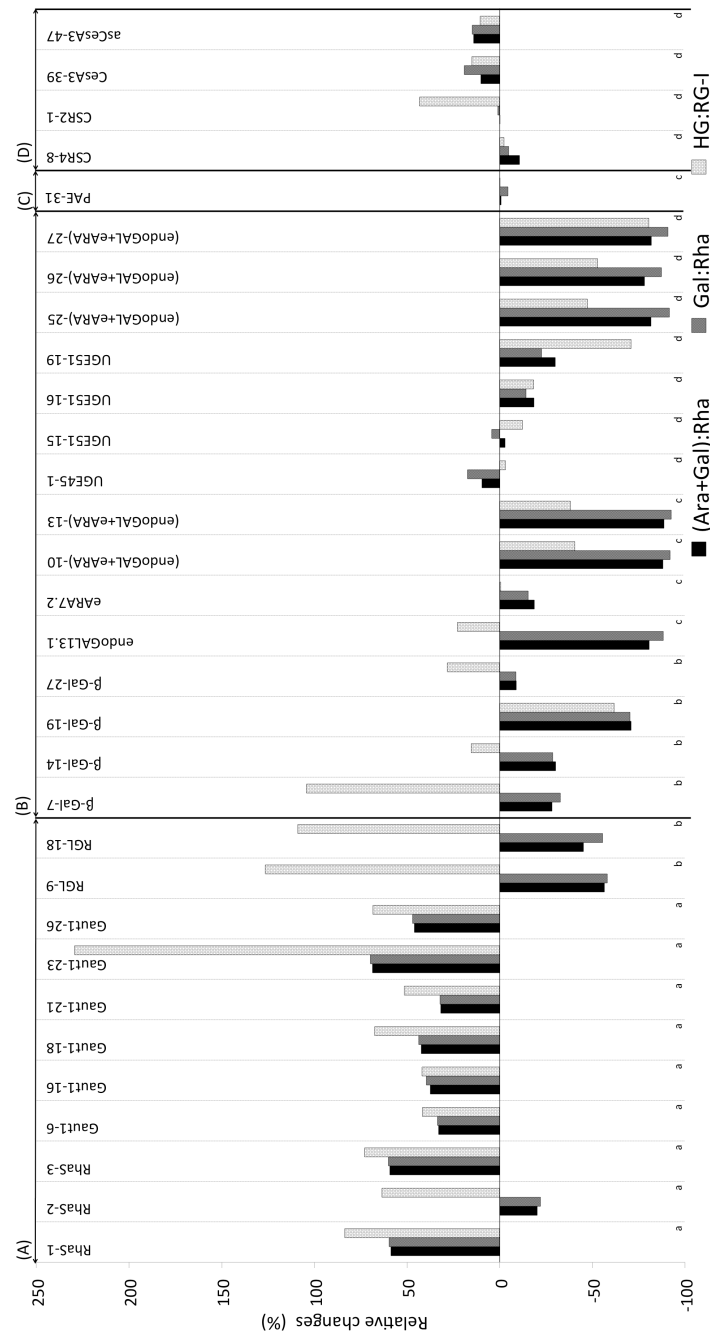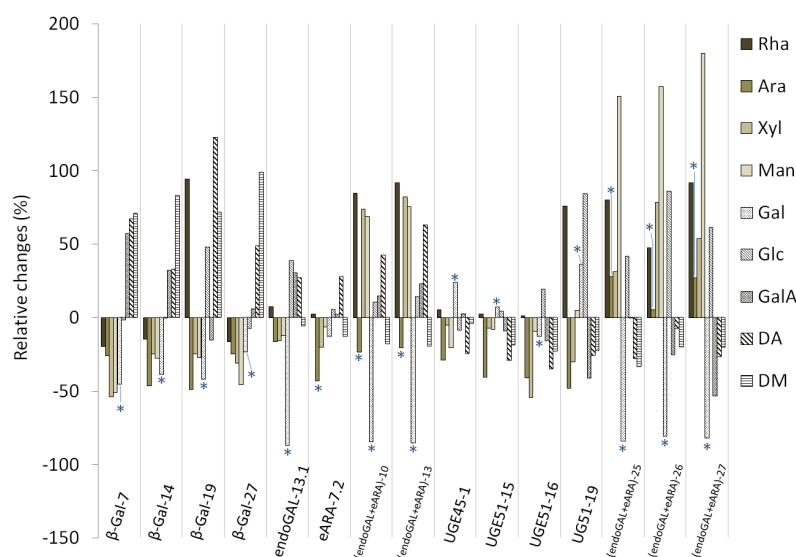
Figure 5.3. Effect of transgenic modifications of (A) pectin backbone, (B) pectin side chains, (C) pectin esterification, and (D) cellulose level on the pectin side chain length ((Ara + Gal):Rha or Gal:Rha ratio) and pectin backbone structure (HG:RG-I ratio) basis on the molar (mol%) monosaccharide composition. The changes of (Ara + Gal):Rha, Gal:Rha and HG:RG-I ratios are relative to the corresponding values of the wild-type potato variety. Transgenic potato lines with wild-type a) Desiree, b) Karnico, c) Posmo, and d) Kardal as background
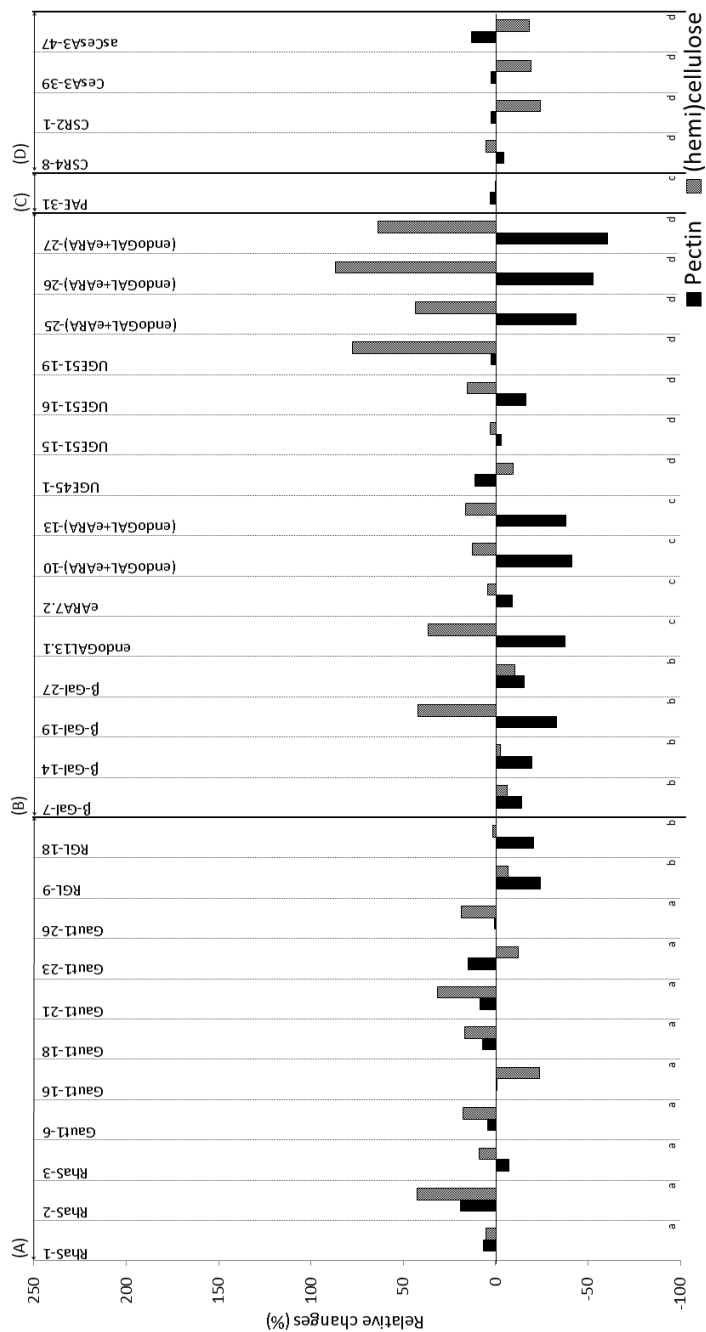
Figure 5.4. Effect of transgenic modifications of (A) pectin backbone, (B) pectin side chains, (C) pectin esterification, and (D) cellulose level on the pectin (Ara, Rha, Gal, GalA) and (hemi)cellulose (Xyl, Man, Glc) content in fresh weight basis (mg/100 g tuber). The changes of pectin and (hemi)cellulose contents are relative to corresponding values of the wild-type potato variety. Transgenic potato mutants with wild-type a) Desiree, b) Karnico, c) Posmo, and d) Kardal as background.

Modification of pectin side chains

The β-Gal, endoGAL, eARA and endoGAL + eARA transgenic lines resulted in a decrease in galactan and/or arabinan side chains (Sørensen et al., 2000; Skjøt et al., 2002; Martín et al., 2005). UGE transgenic lines have been introduced with the aim of elongating galactan side chains in potato (Chapter 4) and Arabidopsis (Mohnen, 2008).

Targeted structure: The Gal content in the CWM for β-Gal, endoGal and endoGal + eARA transgenic lines showed a reduction, as expected (Figure 5.5). The Ara content in eARA and endoGAL + eARA transgenic lines showed reduction in the case of Posmo as background. The reduction in Gal and Ara contents resulting in low ratios of (Ara + Gal):Rha and Gal:Rha indicates the presence of shorter side chains (Figure 5.3B). The previous research with double introduction of (endoGAL + eARA) genes showed a simultaneous reduction in Gal and Ara in the plant cell wall with Posmo as the background (Cankar et al., 2014) and in Arabidopsis mutants (Øbro et al., 2009). The Ara content was unexpectedly higher in the (endoGAL + eARA) -25, -26 and -27 transgenic lines with Kardal as the background (Figure 5.5), which might be caused by the different background of Kardal compared to Posmo.

Figure 5.5. Effect of transgenic modification of pectin side chains in β-Gal (7, 14, 19, and 27), endoGAL-13.1, eARA-7.2, endoGAL + eARA (10, 13, 25, 26, and 27) and UGE (45-1, 51-15, 51-16, 51-19) transgenic lines on monosaccharide contents, acetylation (DA) and methyl-esterification (DM) of cell wall pectin. The changes of monosaccharide contents (mg/100 g tuber), acetylation (DA) and methyl-esterification (DM) are relative to corresponding values of the wild-type potato variety. The asterisk denotes the compound which was affected due to the targeted modification.

The gene encoding UGE affects the UGE enzyme, a part of the UDP-sugar interconversion pathway that changes the UDP-Gal:UDP-Glc ratio (Caffall & Mohnen, 2009). The increase of UDP-Gal from UDP-Glc has been hypothesized to correlate to the synthesis of galactan side chains in potatoes (Oomen et al., 2004a). UGE 45-1 transgenic line showed an elongation of galactan or arabinogalactan side chains by 17% and 10%, respectively. In contrast to UGE 45-1, shorter galactan / arabinogalactan side chains are found for UGE 51-16 (minus 14% and 18%, respectively) and UGE 51-19 (minus 23% and 30%, respectively) compared to wild-type (Figure 5.3B). The enzyme system involved in the UDP-sugars interconversion pathway of UGE transgenic lines may be influenced, resulting in varying levels of UDP-sugars that may affect the correlated cell wall polysaccharides during plant biosynthesis.

Non-targeted structure: The β-Gal transgenic lines, except β-Gal-19 and endoGAL, showed higher HG:RG-I ratios (Figure 5.3B). Our previous research also revealed that β-Gal transgenic lines had a high HG:RG-I ratio in the CWM (Huang et al., 2016). All endoGAL + eARA transgenic lines showed a decreased side chains length and HG:RG-I ratio as well as a lower methyl-esterification in pectin.

All pectin side chain-modifying transgenic lines showed a lower pectin content, except for UGE45-1 and UGE 51-19 (Figure 5.4B). The (hemi)cellulose content always increased after the introduction of the gene encoding endoGAL + eARA (Figure 5.4B), but the level of modification was different for the transgenic lines using different backgrounds (Posmo and Kardal, Figure 5.4B).
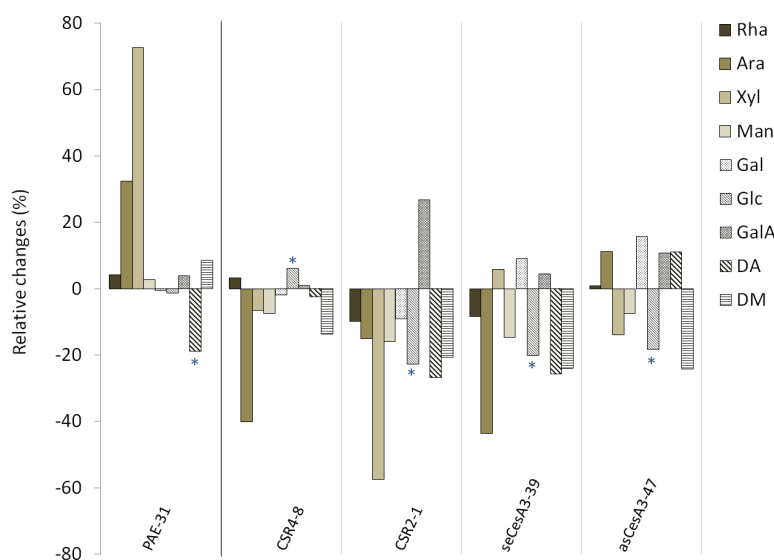
Pectin esterification (DA and DM) was a side effect following pectin side chain modification (Table 5.2). All β-Gal transgenic lines have higher DA and DM of cell wall pectin (Figure 5.5). In contrast to β-Gal transgenic lines, UGE transgenic lines exhibited lower pectin esterification (Figure 5.5). The eGAL + eGARA double construct in the Kardal background also showed a reduction in DA and DM of cell wall pectin. The same genes resulted in a higher DA when transformed into the Posmo background (Figure 5.5). The underlying mechanism remains unclear. The pectin side chain modifying transgenic lines targeting the biosynthesis (e.g., UGE transgenic lines) showed a reduction of pectin esterification, while pectin side chain modifying transgenic lines targeting polymer degradation (e.g., β-Gal) showed on increase in esters. The modified side chain length seems to be accompanied with a change of the net charge of pectin to provide strength to the tissue. However, the alternation of acetylation or methyl-esterification could either lead to higher or lower levels, depending on the transgenic lines.

Modification of pectin esterification

The level of acetylation of pectin can be modified via the expression of the mung bean gene encoding the PAE enzyme in potato tubers (Orfila et al., 2012).

Targeted structures: The overexpression of PAE in potato tubers decreased the level of acetyl groups in CWM by 19% (Figure 5.6). Orfila et al. (2012) reported a 39% decrease in acetyl groups present in potato CWM. The difference in the decrease of acetyl groups might be due to growing conditions.

Non-targeted structure: In contrast to most other transgenic lines, the PAE transgenic line presented no significant adaptation of side chain lengths, pectin backbone structural elements (Figure 5.3C), neither pectin and (hemi)cellulose contents (Figure 5.4C) when compared to wild-type. In contrast, Ara and Xyl levels were markedly higher in the PAE transgenic line (Figure 5.6).

Figure 5.6. Effect of transgenic modification of pectin esterification in PAE-31and cellulose level in CSR (4-8 and 2-1), CesA3-39 and asCesA3-47 transgenic lines on monosaccharide contents, acetylation (DA) and methyl-esterification (DM) of cell wall pectin. The changes of monosaccharide contents (mg/100 g tuber), DA and DM are relative to corresponding values of the wild-type potato variety. The asterisk denotes the compound which was affected due to the targeted modification

Modification of cellulose level

Expression of the genes encoding CesA or asCesA were used to increase or decrease the level of cellulose in potato tubers, respectively (Oomen et al., 2004b). CSR expressed in potato tubers were used for the specific down-regulation of the corresponding CesA genes (Oomen et al., 2004b).

Targeted structure: The reduction in Glc in the CSR2-1, CesA3-39 and asCesA3-47 transgenic lines (Figure 5.6) resulted in an 18%–24% decrease in (hemi)cellulose content (Figure 5.4D). These transgenic lines have been published by Oomen et al. (2004b), but the high amount of residual starch (<90%) in CWM hampered the measurement of cellulose in the cells by colorimetric assay. In our study, we used an improved starch removal procedure, allowing a better evaluation of the constituent monosaccharide composition.

Non-targeted structure: The entire constituent monosaccharide compositions of CSR, seCesA3 and asCesA3 are reported for the first time (Tables 5.1S and 5.2S). Higher HG:RG-I ratios in CSR2-1 (43%), CesA3-39 (19%) and asCesA3-47 (15%) was seen for this category (Figure 5.3D). All transgenic lines showed a reduced DM of the cell wall pectin (Figure 5.6). In combination with a higher GalA level present in CWM, the non-esterified GalA located in the HG structural elements may provide a calcium-pectate complex to maintain the cell wall strength when celluloses decreased. A reduced acetylation for all cellulose targeting transgenic lines except for asCesA3-47 might also improve interaction of pectin to other cell wall polysaccharides providing a strong netwerk within the cell wall architecture.

Overview of the transgenic modification of potato cell wall polysaccharides

The results for all transgenic lines discussed above demonstrate that the introduction of genes into potato tubers can cause modification of both targeted and non-targeted structures. Therefore, based on our results, one should always consider side effects when evaluating transgenic modifications of plant. The effects on cell wall structures of each variety are summarized in Table 5.3. The evaluation provides an overview of each category (A-D) without considering the individual potato transgenic line.

Pectin backbone modifying transgenic lines: Higher HG:RG-I ratios were found in all 11 transgenic lines (Table 5.3). Changing of the pectin backbone introduces side effects to the pectin side chains: 73% of the transgenic lines had longer side chains length compared to wild-type. More than half of the pectin backbone targeting transgenic lines showed either higher or lower acetylation levels of cell wall pectin and the other half of the transgenic lines maintained similar levels of acetylation as wild-type. Lower levels of methyl-esterification were found for 6 transgenic lines. Most of the transgenic lines remained at the

same pectin contents compared to wild-type. The transgenic lines resulting in a modified pectin backbone showed an increase their (hemi)cellulose content (Table 5.3).

Pectin side chains modifying transgenic lines: More than 70% of these lines showed a reduction in side chain lengths. Most of the transgenic lines also resulted in changes in the pectin backbone (low HG:RG-I ratios) when compared to wild-type, which may relate to the presence of shorter side chains (Table 5.3). Based on the results of categories A and B, the (Ara+Gal):Rha, Gal:Rha and HG:RG-I ratios had simultaneously increased or decreased after transgenic modification. The level of methyl-esterification was more influenced in this category than the level of acetylation. A lower pectin content on fresh tuber weight basis tended go along with a higher (hemi)cellulose or similar hemi(cellulose) content than the wild-type. The change of methyl-esterification may contribute the strength of the cell wall structure.

Pectin esterification modifying transgenic lines: In this category, only one transgenic line was available, which had less acetylation in cell wall pectin as expected. Only limited side effects were seen in this category. The low level of acetylation of pectin did not change the cell wall composition and architecture.

Cellulose level modifying transgenic lines: There was a decrease in (hemi)cellulose content in this category. Half of transgenic lines had longer pectin side length with simultaneously higher HG:RG-I ratio than wild-type as found in categories A and B. The cellulose level transgenic lines maintained a same amount of pectin in the tuber.

Modified cell wall architecture: The precise mechanisms behind the modification of level and structure of polysaccharides which are not targeted by the transgenic modification remain unclear. It is hypothesized that a targeted modification of the potato cell wall polysaccharides have a direct effect on the cell wall architecture during tuber development, which may or may not result in a secondary set of changes of the cell wall polysaccharides to compensate for this. The modified cell wall polysaccharides may change the cell wall architecture, which may correlate to the absolute amount of CWM present. A low yield of CWM was found for the pectin backbone transgenic lines (category A, Table 5.2S). In addition, it was noticed that different levels of starch as stored as granules in the amyloplast of the potato tuber (Libessart et al., 1995), could be removed differently, depending on the type of trangenetic modification. This effect was most obvious for the transgenic lines targeting pectin side chains (category B, Table 5.2S), where more residual starch in CWM was found.

Table 5.3. Number of transgenic lines per category being changed on the level of homogalacturonan:rhamnogalacturonan I (HG:RG-I), (Ara+Gal)/Rha, Gal/Rha, acetylation (DA), methyl-esterification (DM), pectin (Ara, Rha, Gal, GalA) content, and (hemi)cellulose (Xyl, Man, Glc) content. Those values obtained from category (A) pectin backbone, (B) pectin side chain, (C) pectin esterification and (D) cellulose level are in comparison to wild-type.

Category	HG:RG-I[a]			(Ara+Gal):Rha[a]			Gal:Rha[a]			DA			DM			Pectin content[b]			(hemi)cellulose content[b]		
	↑	±	→	↑	±	→	↑	±	→	↑	±	→	↑	±	→	↑	±	→	↑	±	→
Pectin backbone[c]	11	0	0	8	0	3	8	0	3	3	5	3	2	3	6	2	7	2	5	5	1
Pectin side chains[d]	4	3	8	0	3	12	1	3	11	8	1	6	4	3	8	0	5	10	8	7	0
Pectin esterification[e]	0	1	0	0	1	0	0	1	0	0	0	1	0	1	0	0	1	0	0	1	0
Cellulose level[f]	2	2	0	2	2	0	2	2	0	0	2	2	0	1	3	0	4	0	0	1	3

↑: more than 15%, ±: within 15%, ↓: more than -15% change compared to wild-type.
[a]HG:RG-I, (Ara+Gal):Rha and Gal:Rha were calculated based on the molar (mol%) monosaccharide.
[b]Pectin and (hemi)cellulose contents were calculated based on the fresh weight basis (mg/100 g tuber).
[c]RhaS, Gaut1 and RGL lines,: in total 11 transgenic lines.
[d]β-Gal, endoGAL, eARA and (endoGAL + eARA) lines: in total 15 transgenic lines.
[e]PAE line: in total 1 transgenic line.
[f]CSR, CesA and asCesa lines: in total 4 transgenic lines.

Conclusions

A new screening strategy to evaluate transgenic potato tubers using the yield of CWM and the sugar composition of the CWM has been introduced. From the sugar composition, various pectin and cell wall characteristic parameters were suggested as powerful indicators of cell wall polysaccharide structure. With the help of these parameters, it was clearly demonstrated that next to the targeted structures, also other cell wall polysaccharides have been modified upon transgenic modification. The screening strategy provides a rapid approach to gain quantitative information for evaluating both targeted and non-targeted modification of potato transgenic lines.

Acknowledgements

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Table 5.1S. Molar monosaccharide composition (mol%), total carbohydrate content, and degrees of acetylation (DA) and methyl-esterification (DM) of cell wall materials from wild-type potatoes (Desiree, Karnico, Posmo, and Kardal) and transgenic lines with modification of (A) the pectin backbone, (B) pectin side chains, (C) pectin esterification and (D) cellulose synthesis.

Category	Name	mol %							Carbohydrate content (% w/w)	DA (%)[b]	DM (%)[b]
		Rha	Ara	Xyl	Man	Gal	Glc[a]	GalA			
Wild-type	Desiree (D)	2	4	2	2	43	38	9	53	14	47
	Karnico (K)	1	6	3	2	28	47	13	65	11	25
	Posmo (P)	1	4	1	1	27	49	17	58	13	45
	Kardal (L)	1	5	2	1	29	45	17	65	23	46
(A)	RhaS-1 (D)	1	4	1	1	44	39	10	57	17	25
	RhaS-2 (D)	2	4	2	2	33	43	14	69	10	36
	RhaS-3 (D)	1	3	2	2	41	42	9	53	19	34
	Gaut1-6 (D)	1	3	2	2	42	41	9	58	16	47
	Gaut1-16 (D)	2	3	1	2	49	33	10	48	18	33
	Gaut1-18 (D)	1	3	2	2	42	40	10	59	15	42
	Gaut1-21 (D)	1	3	2	2	39	44	9	63	15	27
	Gaut1-23 (D)	1	3	1	2	44	33	16	55	11	23
	Gaut1-26 (D)	1	3	1	1	41	44	9	58	14	40
	RGL-9 (K)	1	3	3	1	12	54	26	57	9	44
	RGL-18 (K)	1	6	3	2	12	53	23	59	10	39
(B)	β-Gal-7 (K)	1	5	1	1	17	53	22	59	19	43
	β-Gal-14 (K)	1	4	2	1	19	54	19	58	15	46
	β-Gal-19 (K)	2	3	2	1	16	66	10	57	25	43
	β-Gal-27 (K)	1	5	2	1	25	51	15	69	17	50
	endoGAL-13.1 (P)	1	3	1	1	3	69	22	58	16	42
	eARA-7.2 (P)	1	2	1	1	24	53	18	56	16	39
	endoGAL+eARA-10 (P)	2	3	3	2	5	62	23	50	18	37
	endoGAL+eARA-13 (P)	2	3	3	2	4	63	23	52	21	36
	endoGAL+eARA-25 (L)	2	7	3	3	5	63	17	59	16	30
	endoGAL+eARA-26 (L)	1	5	4	2	5	72	11	69	21	37
	endoGAL+eARA-27 (L)	2	7	4	3	5	71	8	60	17	37
	UGE45-1 (L)	1	4	2	1	35	40	17	62	17	44
	UGE51-15 (L)	1	3	2	1	31	47	15	61	16	37
	UGE51-16 (L)	1	3	1	1	26	54	14	60	15	35
	UGE51-19 (L)	1	2	1	1	29	59	7	84	17	35
(C)	PAE-31 (P)	1	5	2	1	26	48	17	59	10	49
(D)	CSR4-8 (L)	1	3	2	1	29	47	17	61	22	40
	CSR2-1 (L)	1	5	1	1	30	38	24	55	17	36
	CesA3-39 (L)	1	3	3	1	34	39	19	56	17	35
	acCesA3-47 (L)	1	6	2	1	34	37	19	60	25	35

[a]Number of moles of acetyl or methyl groups per 100 moles of galacturonic acid
[b]Glc content after starch correction.

Table 5.2S. Yield of cell wall materials (CWM), individual monosaccharide content of CWM in 100 g of fresh tubers from wild-type potatoes (Desiree, Kanico, Posmo, and Kardal), and transgenics with modification of (A) the pectin backbone, (B) pectin side chains, (C) pectin esterification, and (D) cellulose synthesis.

Category	Name	CWM(%)	Starch(%)[a]	Rha	Ara	Xyl	Man	Gal	Glc[b]	GalA	Carbohydrate content
Wild-type	Desiree (D)	10.3	11.5	10	16	7	8	229	207	54	532
	Karnico (K)	5.9	16.1	6	33	14	11	186	311	89	651
	Posmo (P)	6.0	12.9	5	17	6	6	154	281	107	576
	Kardal (L)	5.1	14.7	5	27	11	7	176	269	111	605
(A)	RhaS-1 (D)	7.7	8.6	7	16	7	7	247	221	61	565
	RhaS-2 (D)	8.2	9.3	13	21	12	10	231	295	105	687
	RhaS-3 (D)	8.0	8.2	6	14	9	8	218	224	51	531
	Gaut1-6 (D)	6.8	8.7	8	16	9	10	242	243	57	585
	Gaut1-16 (D)	9.3	9.8	7	13	4	7	234	159	53	477
	Gaut1-18 (D)	7.9	8.6	7	15	9	11	247	239	62	592
	Gaut1-21 (D)	8.7	9.3	8	17	9	11	248	272	63	628
	Gaut1-23 (D)	9.0	8.8	6	15	7	9	241	180	94	553
	Gaut1-26 (D)	8.8	8.7	7	15	6	8	233	249	58	576
	RGL-9 (K)	6.5	14.0	5	14	12	7	64	296	157	554
	RGL-18 (K)	5.8	13.5	5	28	15	11	69	317	149	594
(B)	β-Gal-7 (K)	4.5	13.6	5	25	7	5	101	305	140	587
	β-Gal-14 (K)	5.1	14.9	5	18	11	8	113	310	118	583
	β-Gal-19 (K)	6.7	16.8	12	17	11	8	107	460	75	690
	β-Gal-27 (K)	6.8	13.9	5	25	10	6	142	287	94	569
	endoGAL-13.1 (P)	6.0	18.4	5	14	5	5	19	390	139	578
	eARA-7.2 (P)	6.0	17.3	5	9	5	6	134	296	109	564
	endoGAL+eARA-10 (P)	6.1	17.0	9	13	10	10	23	310	122	497
	endoGAL+eARA-13 (P)	6.9	15.3	10	13	11	11	22	320	131	517
	endoGAL+eARA-25 (L)	7.9	15.0	10	34	15	17	27	380	110	593
	endoGAL+eARA-26 (L)	6.6	15.3	8	28	20	17	33	499	82	687
	endoGAL+eARA-27 (L)	7.3	17.3	10	34	17	19	31	434	51	596
	UGE45-1 (L)	5.9	12.0	6	19	11	5	217	245	113	615
	UGE51-15 (L)	6.0	15.3	5	16	11	6	188	279	100	605
	UGE51-16 (L)	5.2	14.6	5	16	5	6	153	320	93	598
	UGE51-19 (L)	5.7	16.5	9	14	8	7	239	495	64	837
(C)	PAE-31 (P)	5.6	12.4	5	22	10	6	154	278	111	586
(D)	CSR4-8 (L)	5.1	17.1	6	16	11	6	173	285	112	608
	CSR2-1 (L)	7.5	15.1	5	23	5	6	160	208	140	546
	CesA3-39 (L)	5.4	16.4	5	15	12	6	192	215	116	559
	acCesA3-47 (L)	6.7	21.1	5	29	10	6	203	220	122	596

[a]Starch content of CWM. [b]Glc content after starch correction.

Chapter 6

General Discussion

Motivation and aim of the research

In this thesis study, we aimed to analyze the altered cell wall polysaccharides of potato tubers after transgenic modification. Transgenic modifications are known to affect the targeted polysaccharides, but little is known about the biosynthetic side effects within the whole cell wall architecture. Detailed characterization of transgenetically modified potato cell wall polysaccharides can provide comprehensive structural information about polysaccharides from potatoes and the biosynthetic routes involved. Results of this characterization can subsequently be used for selection of the best potato varieties for a given application, such as cooking, frying and processing. Difficulties associated with the isolation of cell wall material (CWM) have hampered a detailed study of (modified) potato polysaccharides (Sørensen et al., 2000; Jardine et al., 2002). To investigate the modification of potato cell wall polysaccharides, we aimed to develop an improved isolation procedure and further extraction and fractionation protocols to obtain individual polysaccharide populations from potato CWM. Structural parameters have been suggested to be used for investigating a wide range of transgenic modifications of potato cell wall polysaccharides.

Evaluation strategy of transgenic potato

Transgenes encoding plant or fungal enzymes can modify the corresponding enzyme activity in potato, leading to modification of cell wall structures (Øbro et al., 2010). We found that transgenic modification had much broader effects than expected. These side effects have been overlooked most of the times until now. The incomplete information of cell wall polysaccharide structures hinders comparison of transgenic lines. To overcome this problem, an improved strategy for analyzing transgenic potatoes is needed. Both a quick screening as well as an in-depth analysis might be necessary, depending on users' interest and strategy. After selection of candidate transgenic lines by a quick screening method, fractionation and characterization of individual polysaccharide populations from the selected candidate transgenic line can provide detailed information of modified cell wall structures (Figure 6.1).
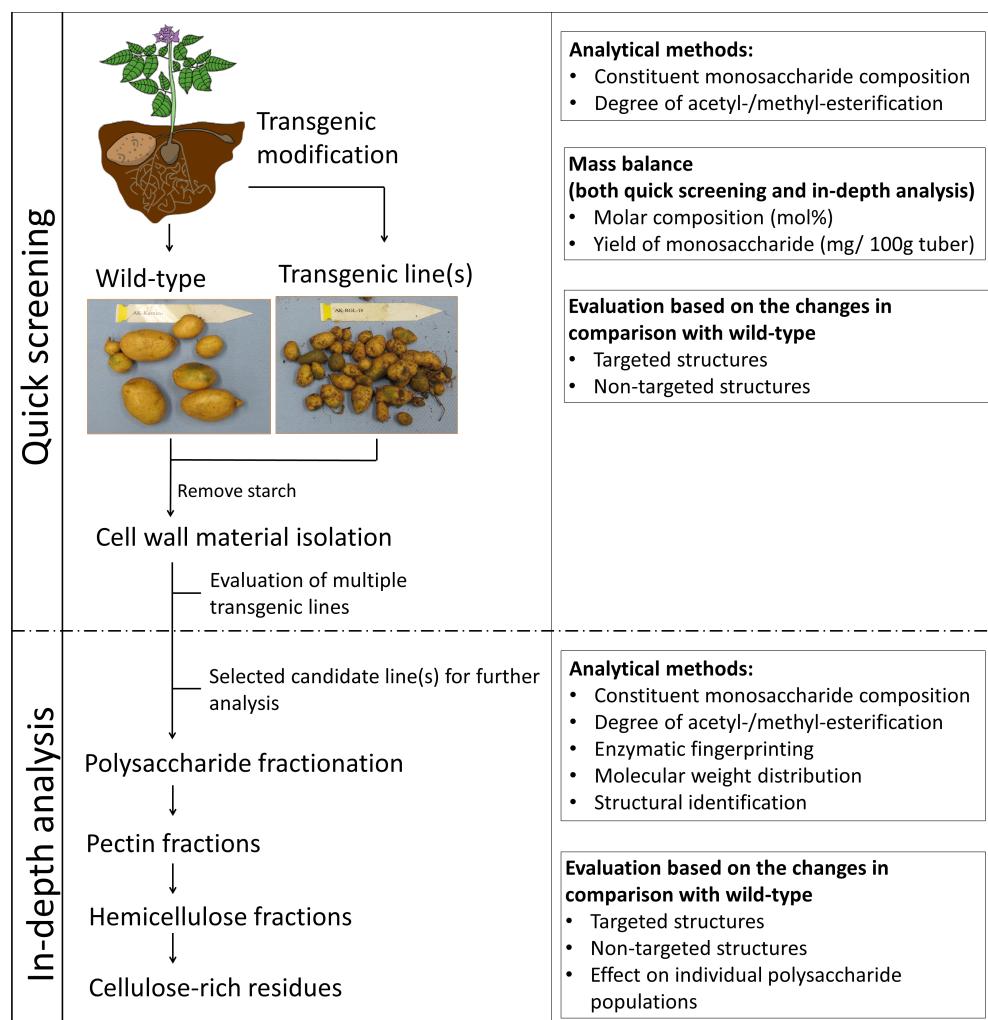
Figure 6.1. Strategy for both quick screening and in-depth evaluation of transgenic potato lines.

Quick screening and evaluation of multiple transgenic lines

To investigate the effects of transgenic modification of potato cell wall polysaccharides, characteristics of each line were evaluated based on polysaccharide's structural parameters (Table 6.1; Chapter 2, 4, 5). With these parameters, a quick description could be made of distinct structural elements within the cell wall polysaccharides. The pectin backbone was quantitatively described by the HG:RG-I ratio indicating the relative importance of the two

main pectin structural elements. Side chain length is being described by the galactose:rhamnose ratio (Gal:Rha) parameter calculating the average length of the galactan side chains. The calculated length could be an underestimation in cases that not all Rha moieties are substituted. Esterification of the pectins is quantified by the commonly used descriptors DA and DM (Chapter 2). Also the absolute amount of total pectin (arabinose [Ara], Rha, Gal plus galacturonic acid [GalA]) and the absolute amount of (hemi)cellulose (glucose [Glc], mannose [Man] plus xylose [Xyl]) (mg per 100g tuber fresh weight) showed to be useful parameters to distinguish between various CWMs. These parameters proved to be essential for a better evaluation of modified cell wall polysaccharose from the transgenic potato. Available transgenic lines were categorized into (A) pectin backbone, (B) pectin side chains, (C) pectin esterification and (D) cellulose level (Chapter 5). The changes (> ±15%) of structural parameters relative to corresponding values of the wild-type as described in chapter 5 are summarized in Table 6.1.

Table 6.1. Transgenic modification of potato cell wall polysaccharides targeting (A) pectin backbone, (B) pectin side chains, (C) pectin esterification, or (D) cellulose level and the percentages of the total transgenic lines within a category leading to a relative change of more than 15% in comparison to the corresponding values of the wild-type.

Transgenic modification (number of transgenic lines)	Structural parameters					
	Gal:Rha (%)	HG:RG-I (%)	DA (%)	DM (%)	Pectin content (%)	(Hemi)cellulose content (%)
A Pectin backbone (11)	100	100*	63	91	36*	55
B Pectin side chain (15)	73*	80	93	80	67*	53
C Pectin esterification (1)	0	0	100*	0	0	0
D Cellulose level (4)	50	50	50	75	0	75*

Abbreviation: HG:RG-I = homogalacturonan:rhamnogalacturonan I; DA = acetylation of cell wall pectin; DM = methyl-esterification of cell wall pectin.
HG:RG-I and Gal:Rha were calculated based on the molar monosaccharide composition. Pectin (Ara, Rha, Gal plus GalA) and (hemi)cellulose (Xyl, Man plus Glc) contents were calculated on the fresh weigh basis (mg/ 100 g tuber).
*transgenic targeted structures

In category A, when the modification was targeting the pectin backbone structure, a modified side chain length was always established as well (Table 6.1). Because pectin side chains may bind to the hemicellulose-cellulose network (Zykwinska et al., 2005), modified side chains are suggested to provide suitable strength for the plant cell wall. Furthermore, the frequently found change in methyl-esterification of cell wall pectin as side effect of a targeted mutation changes the net charge of the pectin backbone, which might provide a necessary non-covalent linkage to other cell wall polysaccharides as well (Vincken et al.,

2003). When the modification was targeting the pectin side chains, a modified backbone was found (category B). It is concluded that Gal:Rha and HG:RG-I ratios and methyl-esterification of pectin are *simultaneously* increased or decreased (Table 6.1), depending on the mutation. Targeting at reducing the acetylation of cell wall pectin had only limited effects on other polysaccharides (category C). The changed abundance of acetyl groups within the cell wall pectin seemed to require less adaptation of the cell wall than other changed polysaccharide structures. Modified acetylation levels of cell wall pectin in categories A and B were caused by the multiple modified pectin structure and suggested a need of the cell wall for an altered interaction in order to support the cell wall architecture. Changes in cellulose levels (category D) also results in modification of the pectin structure (Gal:Rha and HG:RG-I ratios), but did not change the pectin content. The thickness of the cellulose fibrils might be modified after modification of the cellulose level. While our strategy considers both targeted and non-targeted modifications of whole potato tubers, quick screening methods cannot provide detailed information about the individual effects on pectin/xyloglucan polysaccharide populations. Therefore, an in-depth analysis strategy can be followed for the characterization of selected transgenic lines (Figure 6.1).

In-depth analysis of transgenic potato cell wall polysaccharides

Lines targeting pectin-degradation

Following the proposed strategy, introduction of genes encoding β-galactosidase (β-Gal) or rhamnogalacturonan lyase (RGL) enzymes were used as an example for detailed analysis of the effects of transgenic cell wall modification (Chapters 2 & 3).

Targeting galactan side chains: Sequential extraction of different CWM polysaccharide populations and subsequent analysis of the fractions obtained revealed that β-Gal transgenic lines consisted of shorter galactan side chains on the RG-I structural element for buffer-soluble pectin and calcium-bound pectin. The dilute alkali extracts from these transgenic lines, representing pectin interconnected through hydrogen-bonds and esters (Willats et al., 2001), contained longer side chains than found for the wild-type (Chapter 2). Apparently, transgenic modification affects different pectin populations individually. The proposed mechanism of the *in vivo* modification of pectins by the enzyme β-Gal is shown in Figure 6.2A.

Following the β-Gal enzymatic degradation of galactan side chains (Figure 6.2A), the β-Gal transgenic lines might require individual changes of other polysaccharides to support the cell wall structure during tuber development. The Gal released by the enzyme β-Gal enzyme most probably has been reused by the plant itself.
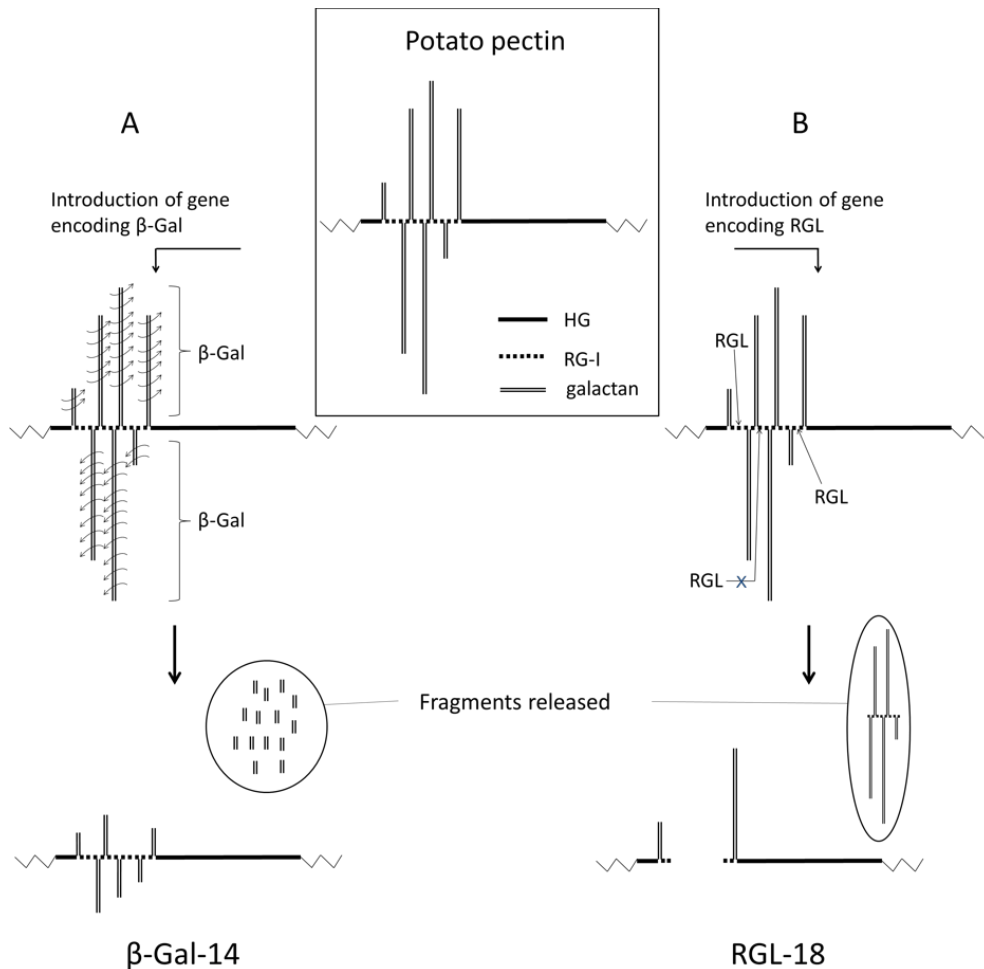
Figure 6.2. Hypothetical scheme for the action of (A) β-galactosidase (β-Gal) and (B) rhamnogalacturonan lyase (RGL) enzymes during *in vivo* modification of potato pectin and the proposed pectin structure in β-Gal-14 and RGL-18 transgenic lines. Individual pectin populations will have different chain lengths of the homogalacturonan (HG), rhamnogalacturonan I (RG-I) and galactan segments.

Potato pectin modification by the β-Gal enzyme and possibly by non-targeted modification (dashed arrows) is shown in Figure 6.3A. The galactan side chains are expected to be degraded by the action of β-Gal (targeted modification, Figures 6.3A and 6.3B). In addition, the pectin structures were unexpectedly modified non-targetedly (Figure 6.3C). Variation in the length of shortened galactan side chains can occur in individual

pectin population. Side effects were seen towards the HG and RG-I elements (minus 30% of HG:RG-I ratio) of the backbone which was also changed with respect to acetylation and methyl-esterification by -31% and -12%, respectively, in comparison to wild-type (Figure 6.3). The mechanisms behind non-targeted changes remain unclear but may help the plant to control the cell wall strength and coherence during growth.
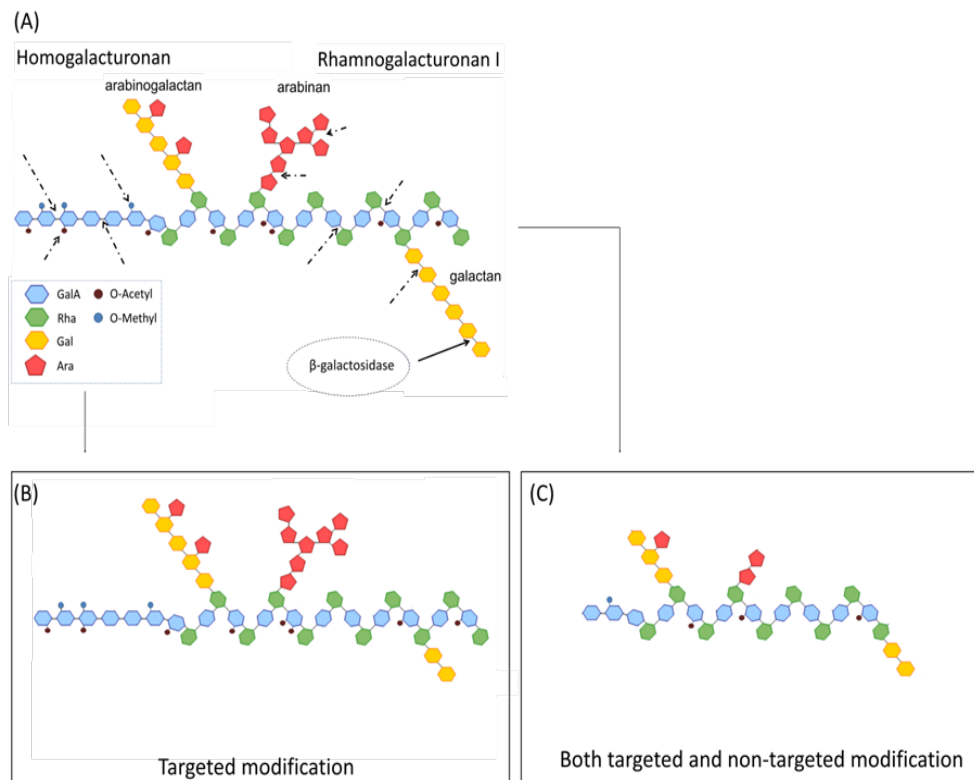
Figure 6.3. Transgenic modification of pectin by β-galactosidase (β-Gal). Schematic representation of (A) pectin of wild-type potato with targeted (arrow) and possible non-targeted modification (dashed arrows) indicated. (B) The hypothesized pectin structure after β-Gal targeted modification and (C) the established pectin structure after both targeted and non-targeted modification.

Furthermore, in addition to pectin modification, the side effects were even extended to xyloglucan structures (Chapter 3). The XXGG-type of xyloglucan is the predominant structure in the potato cell walls (Vincken et al., 1996), but after transgenic modification of pectin structure, especially XXXG-type xyloglucan was present in β-Gal tubers (Chapter

3). The non-covalent absorption of xyloglucan to cellulose fibrils to support the rigidity of cell walls has been widely accepted (Rizk et al., 2000; Cumming et al., 2005). Umemura & Yuguchi (2005) reported that the side chains of xyloglucan can be folded around the xyloglucan backbone. Such a folding in combination with different decorations of the xyloglucan side chains may provide different interaction between xyloglucan and cellulose (Zhang et al., 2011). A modified xyloglucan structure as results of a pectin modification might therefore maintain the rigidity of the cell wall architecture.

Targeting RG-I backbone: Similar HG:RG-I ratios in combination with the low Gal content for all individual pectin fractions obtained from RGL transgenic lines indicates that all pectins were modified in a similar way (Chapter 2; Figure 6.2B). The pectin from the RGL transgenic lines still have relatively long galactan side chains, but fewer of them are present. The released parts of RG-I elements might be recycled as the resource for building up other polysaccharides by the plant itself. It was found that the xyloglucan isolated from the RGL transgenic lines have XXGG-type repeating units like the wild-type, but the main repeating units were present in a different ratio. The precise structure of xyloglucan side chains and their distribution over the glucan backbone and the total length of the backbone determines the conformation of xyloglucan (Lopez et al., 2010) and consequently also the interaction of xyloglucans to other polysaccharides within the (modified) cell wall.

Lines targeting pectin-synthesis

The Golgi-located enzyme uridine diphosphate glucose (UDP-Glc) 4-epimerase (UGE) is known to elongate galactan side chains of the RG-I structural element (Munoz et al., 1996; Seifert et al., 2002; Caffall & Mohnen, 2009). Both UGE45- and UGE51-genes encode for the UGE enzyme in the corresponding transgenic lines, but the enzyme expressed had different effects on the pectin biosynthesis. The UGE45-1 transgenic line resulted in a higher Gal:Rha ratio (elongated galactan side chains), while line UGE51-16 resulted in a lower Gal:Rha ratio (shorter galactan side chains) than the wild-type (Chapter 4). In addition to the targeted modification of pectin side chain, additional modifications were found for the pectin backbone showing a lower acetylation (minus 15%) or higher methyl-esterification (plus 25%) of cell wall pectin and different proportions of xyloglucan subunits representing side effects of UGE transgenic lines (Chapter 4). Those side effects were found for the various polysaccharide populations for the first time for UGE transgenic lines. Previous studies provided limited information on the effect of each UDP-sugar on the interconversion pathways in Arabidopsis and potato (Seifert et al., 2002; Oomen et al., 2004). The enzyme system was changed within the UDP-sugar interconversion pathway, leading to a changed amount of a particular UDP-sugar which leads to a changed assembly of that sugar into the cell wall polysaccharides (Caffall & Mohnen, 2009).

Year to year variability of cell wall polysaccharides from potato tubers

In order to investigate the reproducibility of cell wall composition when growing transgenic potato tubers during different years, wild-type potato varieties and their transgenic potatoes harvested in 2011-2013 as provided by Plant Breeding Wageningen UR and Aviko (Steenderen, the Netherlands) were investigated (unpublished data). A screening of monosaccharide composition was done based on our developed evaluation strategy (Figure 6.1). All structural parameters from transgenic lines were either higher or lower when compared to their wild-types consistently (Table 6.2). The molar monosaccharides composition based structural parameters (HG:RG-I or Gal:Rha ratios) were stable in three years. The pectin and (hemi)cellulose contents were found in similar proportions across the years, but the absolute values were different. The methyl-esterification of pectin (DM) was similar over the years as well as the acetylation of pectin (DA).

This new information indicates that the composition of cell wall polysaccharides were consistent over the year. Although not reported before, also the cell wall polysaccharide characteristics for industrially use potato varieties such as Hansa, Agria, and Innovator exhibited were included in Table 6.2. The Hansa potato variety is used for steam-cooked products. Both Innovator and Agria are often used for the production of French fries.

The variation between the industrial varieties over three years is not different from the wild-type and transgenic lines. In addition, it can be seen from the table that Hansa, compared to Agria, had a low DA that was comparable within the three years. The designed screening protocols resulted in reliable results and allowed an efficient comparison of potato pectin characteristics.

Functional properties of the transgenically modified tubers

Characterization of modified cell wall polysaccharides in transgenic potato was followed by the determination of functional properties, such as the texture of steam-cooked potatoes and stability of potato cubes towards a freeze-thaw cycle (unpublished data).

Effects of modified cell wall polysaccharides on the quality of steam-cooked potatoes. A good cooking quality is an important trait for potatoes. The texture of steam-cooked potatoes varies between non-mealy (firm) and very mealy (loose) (Van Marle et al., 1997a). The cell wall rigidity and strength are influenced by the cell wall composition and structure, which are strongly correlated with the potato texture (Van Marle et al., 1997b; Taylor et al., 2007). Ross (2011) connected the high expression of the pectin methylesterase (PME) gene to a low DM of the cell wall pectin and a more firm cooking behavior of the tuber, which is explained by a *in planta* calcium pectate gel. However, correlations between gene expression, cell wall polysaccharide composition and structure and tuber processing behavior are hardly reported. We compared the cooking behavior of Karnico, Kardal,
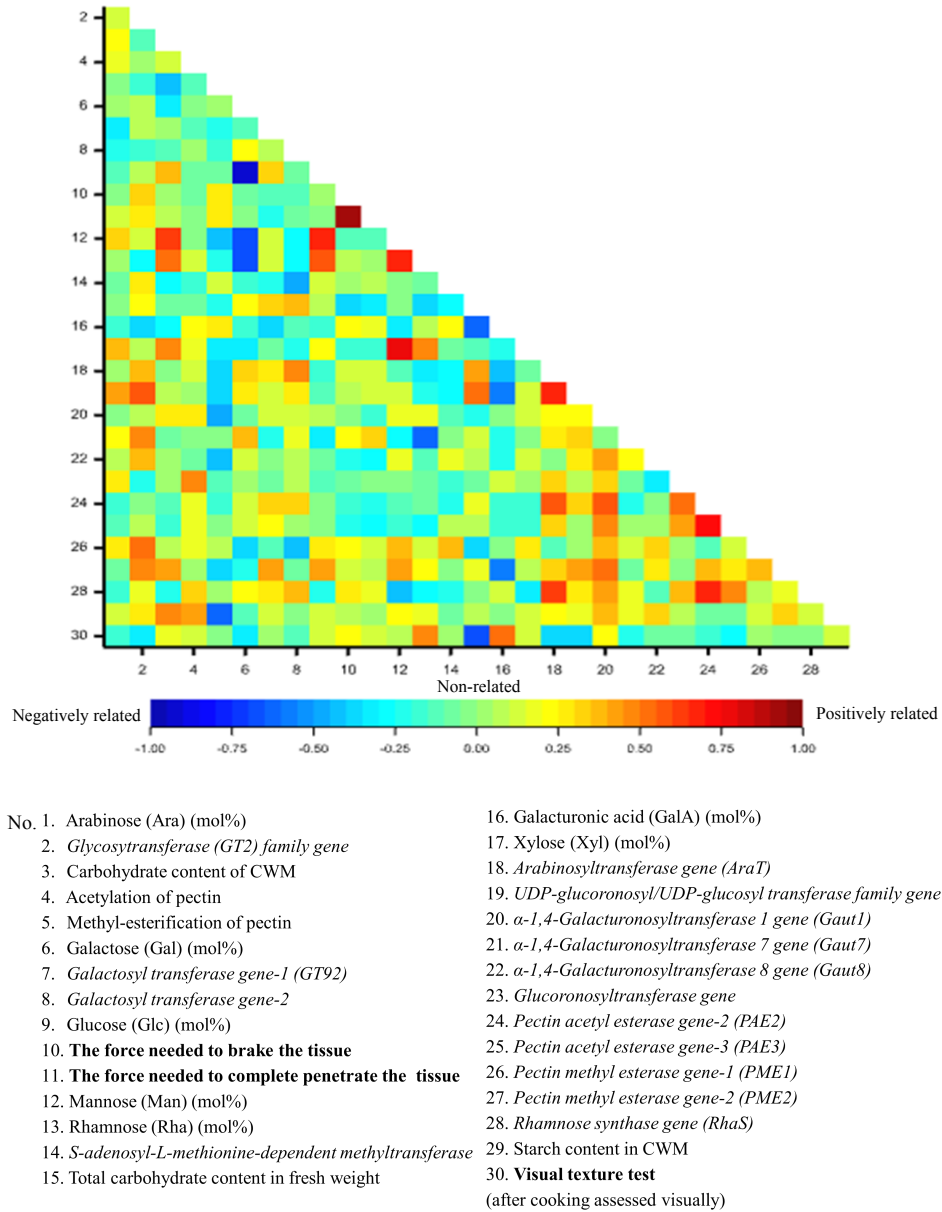
Table 6.2. Structural parameters of wild-type potato varieties (Karnico, Posmo, Kardal and Desiree) and their transgenic lines as well as industrial potato varieties (Harsa, Innovator and Agria) within 2011-2013 years.

Name	Period (years)	Structural parameters					
		Gal:Rha[a]	HG:RG-I[a]	DA	DM	Pectin content[b]	(hemi)cellulose content[b]
Karnico (wild-type)	2011-2013	23-29	4-6	11-19	25-32	293-333	300-353
RGL-9	2011-2013	8-13	12-13	6-12	39-48	227-280	306-364
RGL-18	2011-2013	10-13	9-13	9-11	36-40	239-275	321-362
β-Gal-7	2011-2013	16-21.5	9-15	16-20	38-43	255-288	295-332
β-Gal-14	2011-2013	17-22.2	8-10	11-17	41-52	238-267	302-343
β-Gal-19	2012-2013	8-9	2-3	23-28	43-47	205-219	447-481
β-Gal-27	2011-2013	22-29	7-9	16-21	42-52	245-287	282-323
Posmo (wild-type)	2011-2013	25-31	8-9	11-13	43-47	263-298	286-309
endoGAL13.1	2011-2013	3-4	8-12	12-17	34-45	157-195	364-443
eARA7.2	2011-2013	21-27	7-10	15-17	34-40	243-282	275-344
endoGAL + eARA -10	2011-2013	2-3	4-5	15-18	35-38	144-184	306-348
endoGAL + eARA -13	2011-2013	2-3	5-7	15-22	35-38	162-185	272-401
PAE 31	2011-2013	24-29	6-10	11-15	38-48	270-319	280-301
Kardal (wild-type)	2011-2013	26-36	7-10	19-24	41-48	282-343	275-299
UGE 45-1	2011-2013	32-37	6.7-8	16-21	37-48	323-393	255-367
UGE 51-15	2011-2013	27-34	5.9-8	14-18	33-40	286-337	268-316
UGE 51-16	2011-2013	23-28	5.3-7	14-19	33-38	252-288	307-363
UGE 51-19	2012-2013	22-27	1.4-4	15-21	34-38	314-337	486-524
CSR 4-8	2011-2013	25-31	6.8-9	19-24	35-44	266-334	287-326
CSR2-1	2011-2013	25-32	11.6-12	13-17	32-45	293-349	212-224
seCESA 3-39	2011-2013	30-37	8.5-10	15-18	32-38	292-357	228-236
seCESA 3-47	2011-2013	29-36	8.5-9	21-27	34-41	315-387	210-264
endoGAL + eARA -25	2012-2013	2-4	3.3-6	13-17	23-44	168-191	360-447
endoGAL + eARA -26	2012-2013	3-6	2.9-6	15-24	35-40	140-158	499-564
endoGAL + eARA -27	2012-2013	2-3	1-2	13-18	35-39	124-139	443-489
Hansa	2011-2013	32-44	7-10	12-15	27-35	344-362	216-245
Agria	2011-2013	39-42	7-8	24-31	40-48	356-422	235-259
Innovator	2011-2013	30-35	8-10	18-25	33-35	318-380	260-286

Abbreviation: HG:RG-I = homogalacturonan:rhamnogalacturonan I; DA = acetylation of cell wall pectin; DM = methyl-esterification of cell wall pectin.
HG:RG-I and Gal:Rha were calculated based on the molar (mol%) monosaccharides. Pectin (Ara, Rha, Gal plus GalA) and (hemi)cellulose (Xyl, Man plus Glc) contents were calculated on the fresh weigh basis (mg/ 100 g tuber).

Posmo and Desiree wild type varieties and their transgenically modified potato varieties and used a visual texture test describe the shape of potato tubers after cooking and a texture analyzer to measure the firmness of the cooked potatoes. Upon cooking, a totally different appearance was observed for the wild-type potato varieties and their transgenic lines. After the cooking steps, some transgenic lines had kept firm shape than the wild-type and had easily felt apart. A Pearson's correlation matrix was used to correlate a texture as measured by the texture analyzer (Figure 6.4; No. 10, 11), visual texture test (No. 30) in combination with molar monosaccharide compositions and gene expression to investigate their correlations (Figure 6.4) (Kortstee et al., unpublished data). The texture (No. 30; visual testing) was highly (negatively) correlated to the total carbohydrate content expressed on tuber fresh weight (No. 15), and in the same time highly (positively) correlated to the molar GalA content (No. 16) and intermediately correlated (negative) to galactose (No. 6).

No.
1. Arabinose (Ara) (mol%)
2. *Glycosytransferase (GT2) family gene*
3. Carbohydrate content of CWM
4. Acetylation of pectin
5. Methyl-esterification of pectin
6. Galactose (Gal) (mol%)
7. *Galactosyl transferase gene-1 (GT92)*
8. *Galactosyl transferase gene-2*
9. Glucose (Glc) (mol%)
10. **The force needed to brake the tissue**
11. **The force needed to complete penetrate the tissue**
12. Mannose (Man) (mol%)
13. Rhamnose (Rha) (mol%)
14. *S-adenosyl-L-methionine-dependent methyltransferase*
15. Total carbohydrate content in fresh weight

16. Galacturonic acid (GalA) (mol%)
17. Xylose (Xyl) (mol%)
18. *Arabinosyltransferase gene (AraT)*
19. *UDP-glucoronosyl/UDP-glucosyl transferase family gene*
20. *α-1,4-Galacturonosyltransferase 1 gene (Gaut1)*
21. *α-1,4-Galacturonosyltransferase 7 gene (Gaut7)*
22. *α-1,4-Galacturonosyltransferase 8 gene (Gaut8)*
23. *Glucoronosyltransferase gene*
24. *Pectin acetyl esterase gene-2 (PAE2)*
25. *Pectin acetyl esterase gene-3 (PAE3)*
26. *Pectin methyl esterase gene-1 (PME1)*
27. *Pectin methyl esterase gene-2 (PME2)*
28. *Rhamnose synthase gene (RhaS)*
29. Starch content in CWM
30. **Visual texture test**
(after cooking assessed visually)

Figure 6.4. Pearson's correlation of all traits measured for tubers of transgenic potato plants. Intensity of color indicates strength of correlation. Red color: highly positively related. Blue color: highly negatively related. The name of the genes is given in *italics*, textural traits in bold. The top 3 parameters of cell wall characteristics or genes positively or negatively correlated to visual texture test (No. 30) are listed.

Based on *linear models*,, the cooking behaviour as visually judged can either be predicted by the combination of Gal (neg), acetyl (pos) and residual starch (pos) levels *or* by the combination of the expressed gene levels of Gaut1, Gaut8 and PAE3. Lower Gal levels may indicate a short galactan side chain, which may provide better support of the cell walls and remain a good shape of tubers after cooked. The expression of different genes in the tuber showed a broad range of effects on the visual texture of cooked potato.. A high α-1,4-galacturonosyltransferase1 (Gaut1) gene expression (No. 20) indicates a firm structure for cooked tubers for all transgenic lines, which is in line with the positive correlation of GalA content and texture. Based on the correlations of gene expression, cell wall composition and texture analysis, the tailored cell wall polysaccharides can be used to predict the functional properties of potato tuber.

Freeze-thaw cycle stability: During potato processing, storage methods affect potato texture (Shomer & Kaaber, 2006; Thybo et al., 2006). The freeze-thaw cycle stability of (mashed) potato has been nicely studied by Alvarez et al. (1997, 1998; 2005), but those studies mainly focused on rheological properties rather than on correlating functionality and cell wall polysaccharide composition and structure. Therefore, the freeze-thaw test was performed by moving diced potatoes from storage at 4 °C to storage at −20 °C every 12 hours. The visual performance of potato tubers after freeze-thaw cycle process is shown in Figure 6.5 (unpublished data).
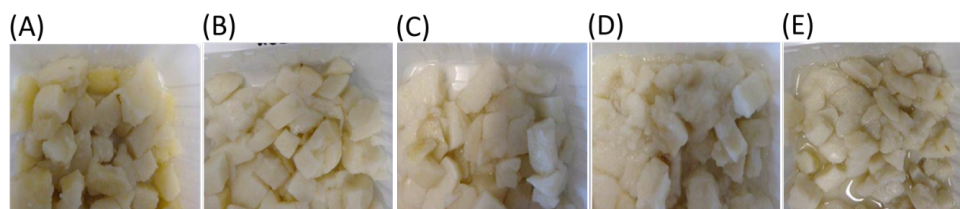
Figure 6.5. Images of potato tuber slices/dices after freeze-thaw cycles for (A) Kardal (wild-type) and its transgenic line (B) UGE45-1, (C) UGE51-16, (D) (endoGAL + eARA)-26 and (E) (endoGAL + eARA)-27.

The most firm dices were found for wild-type (Figure 6.5A) and UGE45-1 (Figure 6.5B). The tuber slices of UGE45-1, (endoGAL + eARA)-26 and -27 transgenic lines after freeze-thaw cycles easily fall apart and showed less firmness (Figure 6.5C-D). These observations seem to correlate with long(er) galactan side chains of pectin as found for wild-type Karnico and the transgene UGE45-1 and shorter galactan side chains found for UGE51-16, (endoGAL + eARA)-26 and -27 transgenic lines (Chapter 4 & 5).

Pectic galactan side chains have been shown to interact with cellulose microfibrils connecting the pectin network with the cellulose-xyloglucan network (Zykwinska et al., 2005). The long galactan side chains present in Karnico and UGE45-1 may so provide more strength to the cell walls and consequently to the tissue and so improve freeze-thaw stability. These preliminary results can used to select potato varieties for frozen potato products.

Does transgenic modification influence non-targeted enzyme in the potato?

Based on our findings, targeted polysaccharides were modified by the introduction of genes encoding specific enzymes, while non-targeted structures were biosynthetically modified unexpectedly as well. These effects result from modifications of the enzyme system in the potato transgenic lines during plant development. To study the endogeneous enzymes present in tuber tissue, enzymes were extracted from wild-type and its transgenic lines to investigate the possible changing on enzyme system after transgenic modification (unpublished results).

Enzymes were extracted from wild-type Karnico and its RGL transgenic lines RGL-9 and RGL-18, by using a high salt potassium phosphate buffer at pH 7.0 and subsequently partly purified using an ammonium sulfate precipitation. After re-solubilisation, the protein extracts were examined for enzyme activities present. The RGL enzyme was not found in Karnico, but this enzyme was found in both RGL-9 than in RGL-18, with lower levels for the latter. RGL-9 and RGL-18 transgenic lines showed higher HG:RG-I ratios (125% and 98%, respectively) than the wild-type (Chapter 5). This nicely correlated to the higher gene express in RGL-9 than RGL-18 (Oomen et al., 2002), which resulted a higher RGL activity in RGL-9 than RGL-18.

An increased pectin methylesterase (PME) activity was found in the extracts from both RGL transgenic lines. The higher PME activities found for the RGL transgenic lines did not correlate with the high methyl-esterification of cell wall pectins as found for these lines (Chapters 2 & 5). Both endo- and exo-polygalacturonases (endoPG and exoPG) were not found in Karnico and two RGL transgenic lines.

After transgenic modification of targeted polysaccharides, the plant itself has been biosynthetically changing the non-targeted polysaccharides to maintain the cell wall architecture. The activity of changed enzyme system may provide an unpredictable fine-tuning on the non-targeted structures during plant development. However, the indirect evidence of changed enzyme systems was found. The plant has been biosynthetically modified the cell wall polysaccharides by the changed enzyme system, but more evidence is still needed.

Research needs for the future

RG-I related research

Potato pectin in both the wild-type and transgenic lines have long galactan side chains (high Gal:Rha ratio), attached to the Rha residues of RG-I backbone. Side-chain length influences cell wall architecture through the interaction between these pectin side chains and cellulose microfibrils (Zykwinska et al., 2005) and changes in the galactan length seems to be responsible for a cascade of modifications within the cell wall as well for changes in functionality of the tuber as is demonstrated in our research. Therefore, a method is needed to evaluate the RG-I structures and the precise length of neutral side chains but current available methods are not fully sufficient for such measurement.

Currently, electron microscopy of plant tissue being immunolabelled with polysaccharide-specific antibodies has been the most promising method over the last two decades for analyzing modified cell wall structures (Knox et al., 1990; Jones et al., 1997; Willats et al., 1998; Cornuault et al., 2015; Leroux et al., 2015). However, by this technique, hardly any information can be obtained addressing the precise structure of these modified polysaccharides.

To investigate the length or distribution of galactan side chains on the RG-I backbone, a method to specifically hydrolyze the RG-I backbone while leaving the side chains connected to the Rha moiety, would be extremely useful. Methods for degrading the RG-I backbone have been described. One approach would be the chemical β-eliminative degradation of the pectin backbone to cleave the α-l-1,2-Rha$p$-α-d-1,4-Gal$p$A linkages within the RG-I backbone, resulting in unsaturated GalA-Rha dimers still carrying side chains of Gal residues linked to the Rha residue (Deng et al., 2006; Deng et al., 2009) as demonstrated for rather linear RG-I from *Arabidopsis thaliana* seed mucilage. The same method was applied for identification of RG-I structure from white cabbage pectin (Westereng et al., 2009). Applied to potato pectin, this approach would yield remnants of the backbone with a wide variety of side chains.

Another approach would be to split the RG-I backbone enzymatically, independently of the presence of (long) galactan side chains. Obviously this is not the case for fungal RGL enzyme (from *Aspergillus niger*) (Mutter, 1997) as used to trangenatically modify potato pectin. Recently, bacterial RGL enzymes from *Bacteroides thetaiotaomicro* have been recognized, being involved in the rather complete degradation of plant polysaccharides (Martens et al., 2011). These RGL enzymes are thought to be more tolerant for substitution of the RG-I. The RGL digestions may end up with remnants of the backbone with a variety of galactan side chains as mentioned for the chemical degradation method. Once the appropriate method for degradation of the potato RG-I backbone has been developed, the available methods like LC-MS and MALDI-TOF MS can be used to investigate these

diagnostic oligomeric fragments. Isomers present among the diagnostic fragments generated could be separated and identified through the use of ion mobility mass spectrometry (Huang et al., 2013) directly or after coupling to e.g. hydrophilic interaction chromatography (Leijdekkers et al., 2015) enabling distinction between RG-I side chains of a similar composition but different in linkage type and/or sequence of the composing sugars.

When the available RG-I degradation and characterization methods have been optimized, a detailed study of pectin structure can provide a good view of cell wall architecture. Consequently, improved knowledge the potato cell wall architecture can provide a better explanation of functional proprieties of transgenic potato tubers. The same methods for RG-I identification can also be used for other plant species.

Interactions of cell wall polysaccharides

Plant cell wall polysaccharides are highly dynamic (Bellincampi et al., 2014). The intermolecular interactions of these polysaccharides are able to maintain cell wall architecture during developmental growth (Cosgrove, 2005). Using imaging techniques would be beneficial for a fundamental study of precise location and interaction within cell wall architecture.

Atomic force microscopy has been used to visualize the ultrastructure of plant cell wall materials (Kirby et al., 1996) and to detect interactions within cell walls (Zhang et al., 2012). However, the information of cell wall composition is not available via atomic force microscopy analysis. Mass spectrometry is a promising technique for characterizing plant cell wall polysaccharides (Bauer, 2012).

Enzymatic fingerprinting in combination with LC-MS or MALDI-TOF MS is a powerful method for identifying plant cell wall polysaccharides (Westphal et al., 2010a; Westphal et al., 2010b). However, mass spectrometry is unable to detect the distribution of cell wall polysaccharides in plant tissue. MALDI or desorption electrospray ionization (DESI) imaging mass spectrometry is an emerging technique that can be combined with precise structural information and the distribution of identified structures (McDonnell & Heeren, 2007). The plant cell wall polysaccharides can be degraded by purified enzymes. Image mass spectrometry can analysis the structure and location of cell wall polysaccharides in plant tissue simultaneously.

High-throughput screening

The structural parameters used for evaluating cell wall polysaccharides have been described in Chapter 5. In this study, we compared the structural parameters of CWMs isolated from 31 transgenic lines and their wild-type background in order to understand the structural modifications of cell wall polysaccharides. However, this method was characterized by limitations associated with a large number of samples for industrial

application (i.e., more than 100 samples). A quick CWM isolation procedure with sufficient starch removal may be necessary for applications related to potato or other high-starch content crops. In addition, a high-throughput scanning method is necessary for analyzing the constituent monosaccharide composition, since the derivatization method might be time consuming. Therefore, microarray analysis may be a useful option for assembling a database that includes gene expression and cell wall characterization.

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Summary

Summary

Potato cell walls are composed of pectin, hemicellulose and cellulose. Cell wall polysaccharides are responsible for the stability, rigidity and flexibility of plant tissue. Pectin, a major component of primary plant cell walls, primarily consists of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). To understand the structure–function relationships of potato cell wall pectin, this study aimed to identify the characteristics of both pectin and other polysaccharides as present in cell wall material (CWM) and of individual polysaccharide populations from wild-type potato varieties and their respective transgenic potato lines.

Chapter 1 gives a general introduction to the fine chemical structures of potato cell wall polysaccharides, the main models of cell wall architecture and the cell wall-degrading enzymes, which include pectinases, hemicellulases and cellulases. In addition, transgenic modification of the cell wall through the heterologous expression of various enzymes from fungal or plant origin that could modify potato cell wall polysaccharides *in planta* is addressed. Transgenic modifications of potato cell wall polysaccharides that targeted pectin structures and cellulose levels are summarised. However, due to unsuccessful starch removal during CWM isolation and incomplete analysis of CWM yield and composition, characteristics regarding the different cell wall polysaccharides from previously-studied transgenic potato lines are hardly available.

CWMs were extracted from the Karnico (wild-type) potato and its transgenic lines that expressed either β-galactosidase or rhamnogalacturonan lyase (**Chapter 2**). Improved starch removal procedures proved to be successful. Pectic polysaccharides were fractionated from CWMs of wild-type potato and its transgenic lines β-Gal-14 and RGL-18. Most β-Gal-14 pectin populations had less galactose (Gal) than wild-type, indicating that the transgenic line had shorter galactan side chains, although the side chain length differed for individual pectin populations. The ratio of HG:RG-I was introduced to evaluate the pectin backbone structure. High HG:RG-I ratios were consistently found in RGL-18 pectic polysaccharide populations. A low level of RG-I segments in combination with lower Gal contents indicated the removal of the galactan-rich RG-I segments in all pectin populations of RGL transgenic lines. In addition, RGL-18 transgenic modification increased the methyl-esterification and lowered the acetylation of pectins present in hot buffer extracts, when compared to wild-type. No effect on pectin esterification was found for β-Gal transgenic lines. Side effects of the mutation generated unexpected changes in the various pectin populations.

The xyloglucan structure was extensively modified after transgenic modification of the pectin structure. Two xyloglucan extracts were obtained from the Karnico and its β-Gal-14 and RGL-18 transgenic lines (**Chapter 3**). The extracts of the Karnico and RGL-18 lines

were mainly comprised of the XXGG-type xyloglucan as represented by XXGG and XSGG as predominant repeating units. In contrast, the XXXG-type xyloglucan was primarily present in the β-Gal-14 4 M alkali extract built up by LLUG repeats, although XXGG type of xyloglucan was present in the 1M alkali extract. Both the RGL and β-Gal transgenic lines had different proportions of xyloglucan building blocks (XSGG/XXGG ratios) than wild-type. After transgenic modification of pectin backbone or pectin side chains, the xyloglucan structures has been biosynthetically modified by plant itself.

Uridine diphosphate (UDP)-glucose 4-epimerase (UGE) catalyses the conversion of UDP-glucose into UDP-galactose, which hypothetically should lead to more galactose being built into the cell wall polysaccharides. CWMs from the Kardal (wild-type) potato and its UGE45-1 and UGE51-16 transgenic lines were isolated, fractionated and characterised (**Chapter 4**). Both the UGE45 and UGE51 genes encoded for UGE enzymes, but the corresponding transgenic lines exhibited different modifications of the galactan side chains and of other cell wall structures. The Gal content of CWM from the UGE45-1 transgenic line was 38% higher than that of the wild-type and resulted in longer pectin side chains. The Gal content present in CWM from UGE51-16 was 17% lower than that of wild-type, which resulted in a slightly shorter galactan side chains for most pectin populations. Both UGE transgenic lines showed a decreased acetylation and an increased methyl-esterification of the cell wall pectin. Side effects were found in the xyloglucan structures of the transgenes as reflected by different proportions of XSGG/XXGG repeating units in comparison to wild-type. Pectin side chain biosynthesis had not only a varying level of galactan side chain modification, but also influenced the structure and possibly the interaction of other cell wall polysaccharides.

In **Chapter 5**, a new screening strategy is introduced to evaluate higher numbers of transgenic potato tubers via CWM yield and sugar composition. A total of four wild-type potato varieties and 31 transgenic lines were evaluated to determine the effects on targeted structures including RG-I or HG pectin backbone elements, galactan or arabinogalactan side chains, acetyl groups of pectin and cellulose levels. Modification of the pectin backbone or pectin side chains in the transgenic lines has either a simultaneous increase or simultaneous decrease of HG:RG-I ratio, side chain length and methyl-esterification of pectin. The pectin esterification transgenic line exhibited only limited side effects. The cellulose level targeted lines had also high HG:RG-I ratios, longer galactan chains and similar pectin content compared to the wild-type, indicative for a less branched pectin backbone with longer side chains. From the monosaccharide composition data, various pectin and cell wall characteristics parameters are suggested as powerful indicators of cell wall polysaccharide structure.

In **Chapter 6,** the achievements of this research are summarised and discussed in the context of potato cell wall architecture. The strategy and outcome of a quick screening

method for multiple transgenic lines and an in-depth analysis of individual pectin and xyloglucan populations for the evaluation of potato CWMs is discussed. Furthermore, the texture of steam-cooked potatoes and the stability of potato cubes after freeze-thaw cycles are correlated with gene expression and cell wall composition in wild-type and selected transgenetically modified potato tubers. CWMs from transgenetically modified potatoes showed different physical properties during processing. In isolated CWMs, acetylation of cell wall pectin, molar Gal levels and starch content were the main parameters that could be related to the texture or firmness of tubers. Tubers from transgenic lines that resulted in shorter pectin side chains felt apart more easily after several freeze-thaw cycles than wild-type tubers and tubers with an increased length of pectin side chains. The modification of both targeted as well as non-targeted structures have now been shown to occur in many different potato transgenic lines, but precise mechanisms and consequences for the cell wall architecture remain unclear. Research performed so far, as well as research needed for getting a better understanding of plant cell wall architecture, is discussed.

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泓泓

About the author

Curriculum Vitae

Jie-Hong Huang was born on February 16, 1983 in Taipei, Taiwan. In 2005, he earned a Bachelor's degree in chemistry from Tunghai University in Taiwan. Then, for the next two years, he earned his Master's degree in chemistry with a specialization in Analytical Chemistry from National Chung Hsing University in Taichung, Taiwan. At the same time, he served as a graduate teaching assistant in the Laboratory of Instrumental Analysis. Under the supervision of Prof. Maw-Rong Lee, he wrote his Master's thesis, which focused on characterizing gibberellic acid and its metabolites in pulse using liquid chromatography–mass spectrometry. His other research involved developing a focused microwave-assisted method for synthesizing room temperature ionic liquids for application in aqueous two-phase system, which can be used to extract and quantify diuretics in human urine. Following one year of military service as a second lieutenant, Jie-Hong worked as an engineer in the Chemicals and Microbiology Division at Intertek Testing Services in Taiwan from 2008 to 2010. From March 2011 to October 2015, under the supervision of Prof. Dr Henk A. Schols and Prof. Dr Harry Gruppen, he served as a PhD researcher in the Laboratory of Food Chemistry at Wageningen University, the results of which appear in the present thesis. In his spare time, Jie-Hong enjoys painting, swimming and trekking.

List of publications

This thesis:

Huang, J.-H., Korstee A., Dees D.C.T., Trindade, L.M.; Schols, H.A., & Gruppen H., (2016). Modification of potato cell wall pectin by the introduction of rhamnogalacturonan lyase and β-galactosidase transgenes and their side effects. *Carbohydr Polym*, accepted for publication.

Huang, J.-H., Jiang, R., Korstee A., Dees D.C.T., Trindade, L.M., Gruppen, H., & Schols, H.A. Modification of potato tuber pectin biosynthesis also affects cell wall xyloglucan structure. *Submitted for publication.*

Huang, J.-H., Korstee A., Dees D.C.T., Trindade, L.M.; Schols, H.A., & Gruppen H., Alteration of cell wall polysaccharides through expression of UDP-Glc 4-epimerase-encoding genes in potato tubers. *Submitted for publication.*

Huang, J.-H., Korstee A., Dees D.C.T., Trindade, L.M., Visser, R.G.F., Gruppen, H., & Schols, H.A. Extended evaluation of cell wall polysaccharide composition in transgenic potatoes. *Submitted for publication.*

Korstee A., **Huang, J.-H.**, Dees D.C.T., Suurs, L., Visser, R.G.F., Schols, H.A., & Trindade, L.M. Keeping shape and firmness of cooked potato tubers are determined by pectic side chain composition and degree of esterification of the backbone. *To be submitted.*

Korstee A., **Huang, J.-H.**, Dees D.C.T., Suurs, L., Dechesne, A., Matser, A., Helmond, M., Visser, R.G.F., Schols, H.A., & Trindade, L.M. Freeze-thaw cycle stability of potato tubers are determined by cell wall polysaccharides. *In preparation.*

Other work:

Leijdekkers, A.G.M., **Huang, J.-H.**, Bakx, E.J., Gruppen, H., & Schols, H.A., (2015). Identification of novel isomeric pectic oligosaccharides using hydrophilic interaction chromatography coupled to traveling-wave ion mobility mass spectrometry. *Carbohydr Res*, *404*(0), 1-8.

Huang, J.-H., Bakx, E.J., Gruppen, H., & Schols, H.A., (2013). Characterisation of 3-aminoquinoline-derivatised isomeric oligogalacturonic acid by travelling-wave ion mobility mass spectrometry. *Rapid Commun Mass Spectrom*, *27*(20), 2279-2285.

Overview of completed training activities

Discipline specific activities

- European polysaccharide network of excellence (EPNOE) conference, Wageningen, The Netherlands, 2011
- Food and biorefinery enzymology[†], VLAG, Wageningen, The Netherlands, 2011
- Plant and seaweed polysaccharides workshop[†], Nantes, France, 2012
- Summer course glycosciences[†], VLAG, Groningen, The Netherlands, 2012
- Advanced food analysis[†], VLAG, Wageningen, The Netherlands, 2013
- Ion trap mass spectrometry training course, Thermo Fisher Scientific, London, UK, 2013
- Mass spectrometry technology days, Waters Corporation, Utrecht, The Netherlands, 2013
- Evaporative light scattering detection training course, JSB, Wageningen, The Netherlands. 2013
- Microscopy and spectroscopy in food and plant sciences, VLAG & EPS, Wageningen, The Netherlands, 2014

General courses

- PhD introduction week, VLAG, Venlo, The Netherlands, 2011
- How to write the world class paper, WU, Wageningen, The Netherlands, 2011
- Scientific publishing, WGS, Wageningen, The Netherlands, 2012
- Reviewing a scientific paper, WGS, Wageningen, The Netherlands, 2013
- Data management planning, WGS, Wageningen, The Netherlands, 2014
- Voice matters-voice and presentation skills training, WGS, Wageningen, The Netherlands, 2014
- Techniques for writing and presenting scientific papers, WGS, Wageningen, The Netherlands, 2014
- Scientific writing, WGS, Wageningen, The Netherlands, 2014

Additional activities

- Preparation PhD research proposal, 2011
- PhD trip[‡], FCH, Singapore and Malaysia, 2012
- PhD trip[‡], FCH, Germany, Denmark, Finland and Sweden, 2014
- STW project meeting, 2011-2015
- BSc/MSc thesis student presentations and colloquia, FCH, 2011-2015
- PhD presentations, FCH, 2011-2015

[†] Poster presentation; [‡] Poster and oral presentations

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences.

EPS: Graduate School for Experimental Plant Sciences.

WU: Wageningen University.

WGS: Wageningen Graduate Schools.

FCH: Laboratory of Food Chemistry.