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Evaluation of both targeted and non-targeted cell wall polysaccharides in transgenic potatoes

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ABSTRACT

In this study, we analyze 31 transgenic lines and their respective untransformed background lines to determine the transgene effects on targeted structures including the pectin components rhamnogalacturonan I (RG-I) and homogalacturonan (HG), neutral side chains (galactan/arabinangalactan), acetylation of pectin, and cellulose level. Modification arising from the pectin backbone- or pectin side chain transgenic lines either increased or decreased the HG:RG-I ratio, side chain length, and methyl esterification of pectin in the tuber cell wall. The pectin esterification transgenic line exhibited only limited side effects. The cellulose level-targeting transgenic lines yielded an unexpectedly high HG:RG-I ratio and longer pectic side chains. These results clearly demonstrate that in effects of a transgene are not restricted to the direct activity of the targeted enzyme but have consequences for the structure of the cell wall matrix. Analysis of whole cell wall structure is therefore necessary to assess the complete effect, direct and indirect, of a transgene.

1. Introduction

Potato tubers are among the most important food crops for human consumption (Camire, Kubow, & Donnelly, 2009). Most potato cultivars are tetraploid with a high level of heterozygosity, providing a huge genetic diversity for breeding purposes (Lehesranta et al., 2005). Conversely, the diverse genetic background presents a challenge for genetic modification of potato and candidate gene selection (Jansky, 2009). In particular, changing the regulation of an endogenous gene or introducing a heterologous gene can specifically modify the cell wall polysaccharides (Bradshaw, 2007), which can in turn be used to investigate potato cell wall architecture. The detailed analysis of different cell wall polysaccharide populations from potato lines expressing different cell wall synthesizing/modifying enzymes can provide information on transgene effects on the entire cell wall. However, such procedures are time-consuming, and only a small number of samples can be analyzed. Therefore, new strategies for evaluating modified cell wall polysaccharides are needed.

Potato pectin is composed of homogalacturonan (HG) and rhamnogalacturonan I (RG-I). In turn, 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 RG-I backbone consists of repeating 4-α-D-GalpA-(1,2)-α-l-rhamnose (Rha) disaccharide units and the side chains are composed of α-L-arabinofuranosyl and/or β-D-galactopyranosyl residues (Albersheim et al., 1996). Lerouxel et al. (2002) reported on the characterization of xyloglucan structures in mutants lines; however, limited information about the complete cell wall polysaccharides has been presented.

Plant cell wall materials (CWM) can be modified by the expression of hydrolytic enzymes *in vivo* to modify targeted structures (Øbro, Hayashi, & Dalgaard Mikkelsen, 2010). The modification of plant cell walls *in planta* has mainly been characterized by microscopy analysis combined with antibody immunolabeling (Jones, Seymour, & Knox, 1997; Knox, Linstead, King, Cooper, & Roberts, 1990; Willats, Marcus, & Knox, 1998) and Fourier transform infrared microspectroscopy (Chen et al., 1998). These microscopy spectra, in combination with molar sugar compositions, can provide qualitative evidence of the structure of modified CWM in transgenic lines (Oomen, Dao-Thi, et al., 2004). Whereas

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Moller et al. (2007) described a high-throughput microarray for screening cell wall polymers, the antibodies necessary for immunolabeling are expensive and not readily available. Additionally, this method can only be used semi-quantitatively. The transgenic effects on potato tubers and other non-modified cell wall polysaccharides have not always been sufficiently characterized (Øbro et al., 2009; Martín et al., 2005; Oomen, Dao-Thi et al., 2004; Sørensen et al., 2000) [12, 14–16] as the focus was usually limited to monitoring targeted structures. Therefore, little is known regarding the entire range of effects on other cell wall polysaccharides, hampering the complete evaluation of potato transgenic lines.

Pectin transgenic lines exhibit specific effects on pectic structures. The side chain modification (β-galactosidase [β-Gal]) transgenic lines exhibited shortened galactan side chains but also showed distinct alterations in the pectin backbone, pectin esterification, and xyloglucan structures (Huang et al., 2016b; Huang, Jiang, et al., 2016). Pectin backbone-modification in rhamnogalacturonan lyase (RGL) transgenic lines also resulted in side effects altering pectin esterification (Huang et al., 2016b). A detailed view of transgene-mediated changes can be useful; although sufficient time and sufficient potato transgenic line material might not be available for the initial screening stage. Therefore, a brief but careful examination including cell wall yield, sugar composition, and pectin characteristics such as HG:RG-I or galactose (Gal):Rha ratios can provide an improved screening output as well as detailed information on modified cell wall polysaccharides (Huang et al., 2016b).

In this study, we evaluated the cell wall structure of a series of 31 transgenic lines and respective untransformed background lines with respect to targeted 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 also re-examined by the proposed evaluation method to provide information currently lacking on non-targeted polymers.

2. Materials and methods

2.1. Transformation of potato cultivars and transgenic lines

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

The following transgenic lines and respective wild-type potatoes (Table 1) were analyzed: (1) Desiree and derived transgenic lines expressing galacturonosyl transferase1 (Gaut1) (Atmodjo et al., 2011) or RhaS (Reiter & Vanzin, 2001); (2) Karnico and transgenic lines expressing RGL (Huang et al., 2016b; Oomen et al., 2002) or β-Gal (Huang et al., 2016b; Martín et al., 2005); (3) Posmo and transgenic lines expressing *endo*-1,4-β-D-galactanase (endoGAL) (Sørensen et al., 2000), *endo*-α-1,5-L-arabinanase (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 overexpressing cellulose synthase (CesA), knock-down of cellulose synthase (asCesA), class-specific regions of cellulose synthase (CSR) (Oomen, Tzitzikas, et al., 2004), UDP-glucose (Glc) 4-epimerase (UGE) (Oomen, Dao-Thi, et al., 2004), 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 1.

As the Gaut1 and RhaS transgenic lines have not been previously described, the cloning procedure is briefly described below. The procedure used for other transgenic lines are referred to in Table 1. The double construct (endoGAL + eARA) has been previously studied in the Posmo background (Cankar et al., 2014). In the current study, both genes were also introduced into the Kardal background. The available information for these and the other potato transgenic lines obtained from previous research is summarized in Table 1.

2.2. Cloning of RhaS and Gaut1 overexpressing constructs

We utilized the Gateway® cloning system to overexpress the *RhaS* or *Gaut1* gene in potato. The coding sequences of the genes were cloned from tuber cDNA from the potato cultivar Kardal and placed under the control of the cauliflower mosaic virus (35S) promoter (Odell, Nagy, & Chua, 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.

2.3. Transformation of RhaS and Gaut1 transgenic lines

Transformation of the binary constructs was performed as described by Visser, Stolte, and Jacobsen (1991). The potato cultivar Desiree was used for the transformation experiments. Potato stem segments were co-cultivated for 3 days with *Agrobacterium tumefaciens* carrying RhaS or Gaut1 plasmids. 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.

2.4. Transgenic-targeted polymers and side effects

Table 2 summarizes the potato transgenic lines based on modification of (A) the 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. In Table 2, along with the targeted modifications marked by "X", empty cells represent possible side effects.

2.5. Potato tuber preparation

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

2.6. Isolation of CWM from potato tubers

To promote starch gelatinization, 5 g dry material was added to 25 mL sodium acetate buffer (pH 5.2) and incubated at 80 °C for 30 min. The detailed protocol has been reported previously (Huang et al., 2016b). 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 pellet was then lyophilized, yielding the CWM.

2.7. Determination of constituent monosaccharide composition

The CWM and obtained fractions were analyzed to determine the constituent monosaccharide composition using a prehydrolysis

Table 1
Wild-type potato varieties (Karnico, Posmo, Kardal, and Desiree); their transgenic lines targeting (A) the pectin backbone, (B) pectin side chains, (C) pectin esterification, and (D) cellulose levels; and reported changes in cell wall polysaccharides.

Category	Transgene (symbol)[a]	Wild-type	Lines	Observed modification to structures	Information available			References
					CWM	Fresh weight	Pectincharacterization	
A	UDP-rhamnose synthase (RhaS)	Desiree	1, 2, 3	Increased RG-I elements in the pectin backbone[b]	No	No	No	Not available[c]
	Galacturonosyl transferase1 (Gaut1)	Desiree	6, 16, 18, 21, 23, 26	Increased HG elements in the pectin backbone[b]	No	No	No	Not available[c]
	Rhamnogalacturon lyase (RGL)	Karnico	9, 18	Shorter RG-I elements in the pectin backbone	Yes	Yes	Yes	Huang et al. (2016b), Oomenet al. (2002)
B	β-galactosidase (β-Gal)	Karnico	7, 14, 19, 27	Shorter galactan side chains of RG-I	Yes	Yes	Yes	Huang et al., (2016a), Martín 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-arabinanase (eARA)	Posmo	7.2	Shorter arabinan side chains of RG-I	Yes	No	No	Skjøt et al. (2002)
	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)
	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	Elongated galactan side chains of RG-I	Yes	Yes	Yes	Chen et al. (1998), Huang et al. (2016a)
C	Pectin acetyl esterase (PAE)	Posmo	31	Lower acetyl substrates of HG	Yes	No	Yes	Orfila et al. (2012)
D	Sense-cellulose synthase (CesA)	Kardal	39	Increased cellulose levels	Yes	No	No	Oomen, Tzitzikas, et al. (2004)
	Anti-sense cellulose synthase (asCesA)	Kardal	47	Decreased cellulose levels	Yes	No	No	Oomen, Tzitzikas, et al. (2004)
	Class-specific regions of cellulose synthase (CSR)	Kardal	4–8, 2–1	Decreased cellulose levels	Yes	No	No	Oomen, Tzitzikas, et al. (2004)

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) and Gaut1 (Atmodjo et al., 2011).

[c] New transgenic lines in potato.

Table 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 the 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		
	Rhamnogalacturonan 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		
	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).

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 the sugars released were analyzed as their volatile alditol acetates by gas chromatography as described (Huang et al., 2016b). The total uronic acid content was determined using an automated-m-hydroxydiphenyl assay (Thibault, 1979) and was attributed to GalA. The CWM starch content 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 Gal:Rha or (Ara + Gal):Rha molar ratios can be used to determine the galactan or arabinogalactan side chain lengths, 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).

2.8. Degree 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, Schols, & Pilnik, 1986). The degree of acetylation and methyl-esterification are calculated as moles methyl esters or acetyl groups per 100 mol GalA. One mole GalA can carry only one mole methyl esters and two moles acetyl groups.

2.9. Strategy for evaluating CWM in transgenic potatoes

The strategy used in this study: first, CWMs were isolated from wild-type potatoes and their transgenic lines. Second, the constituent monosaccharide composition and pectin esterification of CWMs were analyzed. Based on the molar monosaccharide compositions, the side chain length was calculated using Gal:Rha and (Ara + Gal):Rha ratios. The HG:RG-I ratio provides structural information regarding the pectin backbone. The monosaccharide compositions were then used to determine the monosaccharide yield from fresh potato tubers to understand the effect of the transgene on the complete tuber. The DA and DM of pectin were included in the analysis. The pectin (sum of Ara, Rha, Gal, and GalA) and (hemi)cellulose (sum of Xyl, Man, and Glc) contents in the fresh weight potato (mg/100 g tuber) provided the polysaccharide proportions after the transgenic modification of cell wall polysaccharides.

The effect of the modifications in transgenic potato transgenic lines was measured against the corresponding values of the wild-type. The potato transgenic lines were evaluated for effects on both the specifically targeted structures as well as for possible side effects.

3. 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 1). Our strategy not only included the evaluation of modified targeted structures, but also considered side effects towards other cell wall polysaccharides (Table 2). Detailed information on molar composition, individual CWM monosaccharide content (mg/100 g tuber), DA and DM of cell wall pectin for all wild-type and transgenic lines is available in Supplementary Tables 1S and 2S.

Most potato CWMs were found to be rich in Glc and Gal. In contrast, 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 (Section 2.9) and to simplify the comparison, we only presented deviations for the transgenic lines from the wild-type values. Each category of transgenic modification is discussed separately below.

3.1. Modification of the pectin backbone

RhaS catalyzes the conversion of UDP-Glc to UDP-Rha (Reiter & Vanzin, 2001). In Arabidopsis tissue, UDP-Rha has been correlated to the amount of Rha in the cell wall during plant biosynthesis of RG-I elements in the pectin backbone (Reiter & Vanzin, 2001). Gaut1 is involved in synthesizing HG by inserting GalA into pectin HG elements in Arabidopsis (Atmodjo et al., 2011). Introduction of the gene encoding 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., 2016b).

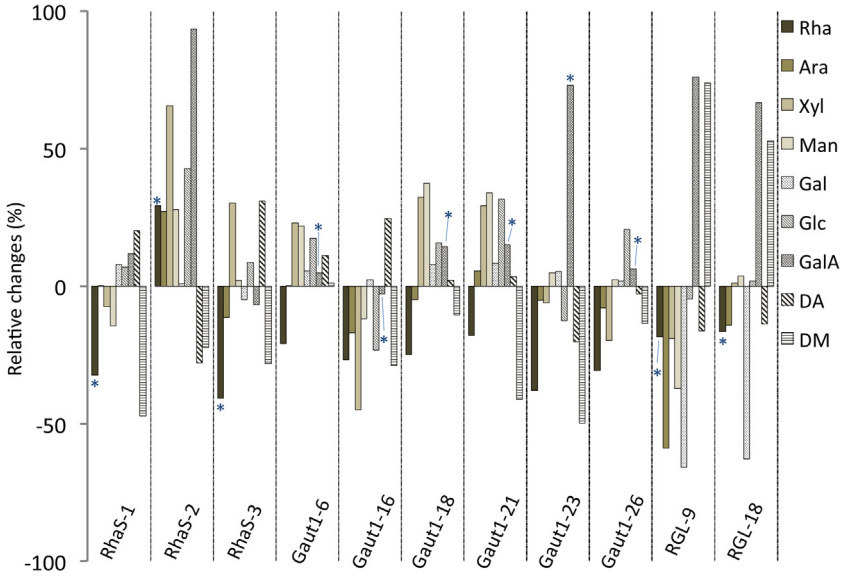

Fig. 1. Effect of transgenic modification of the pectin backbone in RhaS (1, 2, and 3), Gaut1 (6, 16, 18, 21, 23, and 26) and RGL (9 and 18) transgenic lines on monosaccharide contents and acetylation (DA) and methyl-esterification (DM) of the cell wall pectin. The changes of monosaccharide contents (mg/100 g tuber), DA, and DM are relative to the corresponding values of the wild-type potato variety. Asterisks denote the compounds that were affected owing to the targeted modification.

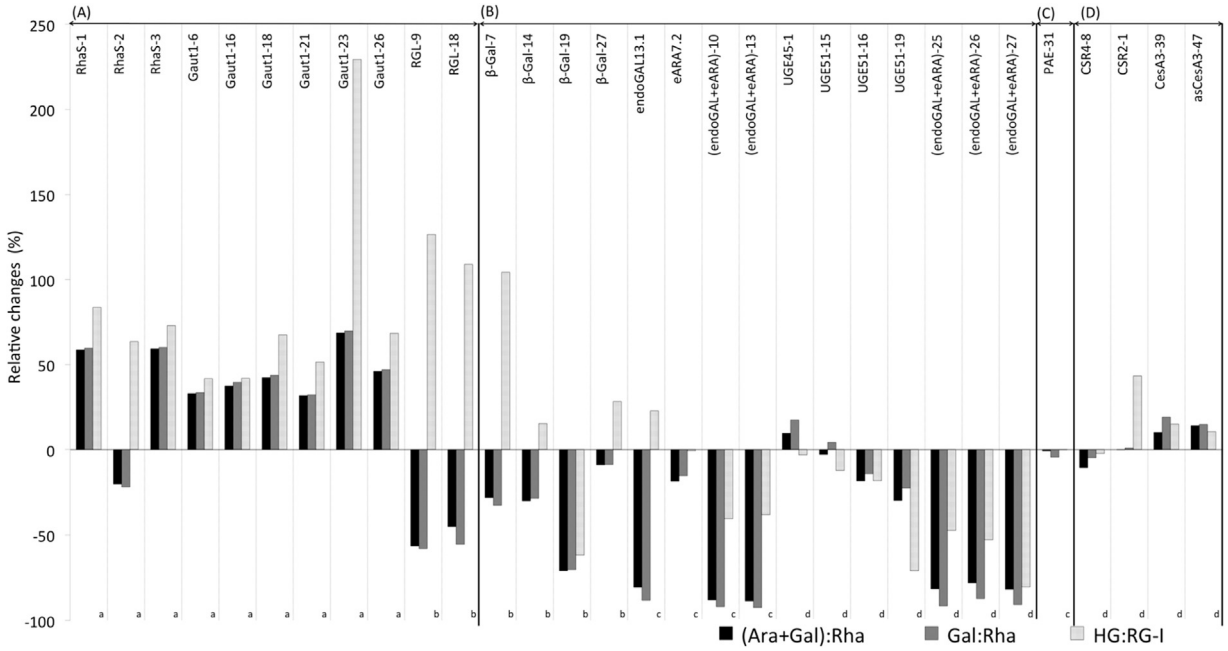

Fig. 2. Effect of transgenic modifications of (A) the pectin backbone; (B) pectin side chains; (C) pectin esterification; and (D) cellulose level on the pectin side chain length ([Ara + Gal]:Rha or Gal:Rha ratio) and pectin backbone structure (HG:RG-I ratio) based on the molar (mol%) monosaccharide composition. The changes of (Ara + Gal):Rha, Gal:Rha, and HG:RG-I ratios shown are relative to the corresponding values of the wild-type potato variety. The wild-type background of the transgenic potato lines is shown as (a) Desiree, (b) Karnico, (c) Posmo, and (d) Kardal.

3.1.1. Targeted structure

In Fig. 1, 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 increased content (plus 29%, Fig. 1). To date, the RhaS gene has only been introduced into the genome of Arabidopsis and only differences of less than 5% in the molar sugar composition of Rha have been shown; no information on the CWM monosaccharide content was 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 transgenic lines on the Rha content, the HG:RG-I ratios from all RhaS transgenic lines were approximately 50% higher than those in wild-type potatoes (Fig. 2A), indicating the presence of relatively more HG structural elements than RG-I segments for all three transgenic lines. After the transgenic modification targeting on RG-I backbone, the physical strength of the cell wall polysaccharides might be different for RhaS 1, 2, and 3 transformants during plant growth. Therefore, the plant might change the cell wall architecture independently for a better structure or strength during

growth. Such mechanisms are still unclear, but might explain the different monosaccharide composition of RhaS lines. Therefore, the further study still needed in the future, but this manuscript indicates the side effect of transgenic modification, which has usually been ignored in the previous study.

The Gaut1 transgenic lines showed an increase in GalA content (mg/100 g tuber) in the CWM ranging from 5%–15%, except for Gaut1-23 (plus 73%) and Gaut1-16 (minus 7%, Fig. 1). The higher ratios of HG:RG-I present in the Gaut1 transgenic lines (Fig. 2A) are caused by both a higher GalA and a lower Rha content (Fig. 1), indicating a reduction of RG-I elements in the pectin backbone. Previous studies showed that Gaut1 catalyzes the transfer of GalA from UDP-GalA to HG (Reiter & Vanzin, 2001; Sterling et al., 2006), however, information is missing regarding the total GalA content in the plant tissue thus making it impossible to judge the effects of Gaut1 enzyme function on cell walls in these studies.

The RGL transgenic lines showed reduced Rha content in CWM (Fig. 1), possibly due to cleavage of the RG-I elements (illustrated by a high HG:RG-I ratio, Fig. 2A). The reduction of Ara and Gal content in CWM (Fig. 1) resulted in low (Ara + Gal):Rha or Gal:Rha ratios when compared to wild-type (Fig. 2A). The release of neutral side chain-rich RG-I in RGL transgenic lines has been confirmed by the detailed characterization of isolated pectin fractions (Huang et al., 2016b). Although the fractionation and characterization of pectin polysaccharides provide clear advantages toward the recognition of all modifications in individual pectin fractions, our proposed screening strategy is more efficient when evaluating multiple transgenic lines.

3.1.2. Non-targeted changes

Non-targeted structures were also addressed to examine the comprehensive effect of transgenic potato tuber modification. In Fig. 1, the bars without an asterisk represent non-targeted monosaccharides; changes in 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 (Fig. 2A). Conversely, RhaS-2 showed shorter galactan (Gal:Rha ratio: 18) and arabinogalactan ([Gal + Ara]:Rha ratio: 16) side chains than the wild-type. Upon calculating the deviations from the wild-type Gal:Rha ratio, the transgenic lines showed approximate 60% increase in the ratio for RhaS-1 and RhaS-3 and a 22% decrease for RhaS-2, respectively (Fig. 2A). The average increases in chain length for RhaS-1 and -2 but not for RhaS-3 correlate well with the deviations found in the Rha content.

Positive Gal:Rha and (Ara + Gal):Rha deviations in comparison to wild-type were found for all Gaut1 transgenic lines, ranging from 35% to 70%, whereas average chain length increases could readily be correlated to reduced Rha levels (Fig. 1). Furthermore, significant changes of DA in both increased and decreased (Fig. 1) occurred in all transgenic lines, although these were not correlated to GalA change. All RhaS and Gaut1 transgenic lines exhibited lower cell wall pectin DM (Fig. 1). However, the mechanisms underlying such side effects remain unclear.

Another way of judging the modification of cell wall polysaccharides is to calculate the pectin content (Ara, Rha, Gal + GalA) and (hemi)cellulose content (Xyl, Man + Glc) based on the fresh weight of the potato (mg/100 g tuber). The pectin and (hemi)cellulose contents may support us to recognize changes in both targeted and non-targeted cell wall polysaccharides. The transgenic pectin and (hemi)cellulose contents in comparison to the corresponding values of wild-type varieties are shown in Fig. 3A. Here, we found that RhaS and Gaut1 transgenic lines had higher (hemi)cellulose contents, except for Gaut1-16 and Gaut1-26 (Fig. 3A). RGL transgenic lines resulted in lower pectin contents but had limited effect on (hemi)cellulose contents (<10%, Fig. 3A).

3.2. Modification of pectin side chains

The β-Gal, endoGAL, eARA, and endoGAL + eARA transgenic lines exhibited a decrease in galactan and/or arabinan side chains (Martín et al., 2005; Sørensen et al., 2000; Skjøt et al., 2002). UGE transgenic lines have also been introduced with the aim of elongating galactan side chains in potato and Arabidopsis (Huang et al., 2016a; Mohnen, 2008).

3.2.1. Targeted structure

The CWM Gal content for β-Gal, endoGal, and endoGal + eARA transgenic lines was reduced compared to their respective wild-types (Fig. 4), as expected. The Ara content in the eARA and endoGAL + eARA transgenic lines is lower when compared to the Posmo background. These reduction resulted in low (Ara + Gal):Rha and Gal:Rha ratios and indicated the presence of shorter side chains (Fig. 2B). In previous studies, double introduction of (endoGAL + eARA) genes yielded a simultaneous reduction in Gal and Ara in the plant cell wall in a Posmo background (Cankar et al., 2014) as well as in Arabidopsis mutants (Øbro et al., 2009). The (engoGAL + eARA) −10 and 13 transgenic lines were using Posmo as background as indicated in the legend of Fig. 3. Conversely, the Ara content was elevated in the (endoGAL + eARA) −25, −26, and −27 transgenic lines in a Kardal background (Fig. 4); this discrepancy might be caused by the different genetic background of these varieties.

UGE comprises a part of the UDP-sugar interconversion pathway that modifies 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, Dao-Thi, et al., 2004). 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 (Fig. 2B). It is possible that the enzyme system involved in the UDP-sugar interconversion pathway might be affected within UGE transgenic lines, resulting in varying levels of UDP-sugars that might in turn impact the associated cell wall polysaccharides during plant biosynthesis.

3.2.2. Non-targeted structure

The β-Gal transgenic lines, except for β-Gal-19 and endoGAL, showed higher HG:RG-I ratios (Fig. 2B). Our previous research also revealed that β-Gal transgenic lines exhibited a high HG:RG-I ratio in the CWM (Huang et al., 2016b). 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 (Fig. 3B). The content of (hemi)cellulose consistently increased after expression of the genes encoding endoGAL + eARA (Fig. 3B); however, the level of modification varied for different backgrounds (Posmo and Kardal, Fig. 3B).

Pectin esterification (DA and DM) also revealed a side effect following pectin side chain modification (Table 2). All β-Gal transgenic lines exhibited a higher DA and DM of cell wall pectin (Fig. 4). In contrast to the β-Gal transgenic lines, UGE transgenic lines demonstrated lower pectin esterification (Fig. 4). The eGAL + eGARA double construct on the Kardal background also showed a reduction in DA and DM of cell wall pectin but resulted in a higher DA when transformed onto the Posmo background (Fig. 4). However, the underlying mechanism remains unclear. The pectin

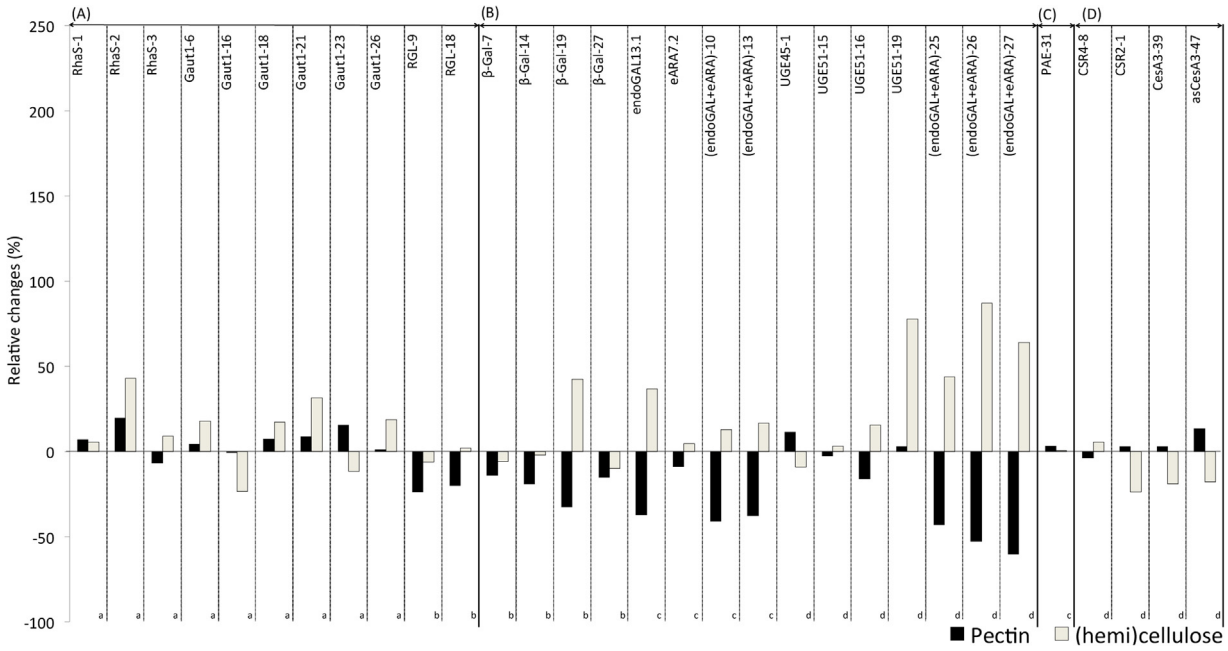

Fig. 3. Effect of transgenic modifications of (A) the pectin backbone; (B) pectin side chains; (C) pectin esterification; and (D) cellulose level on the pectin (Ara, Rha, Gal, and GalA) and (hemi)cellulose (Xyl, Man, and Glc) content by fresh weight basis (mg/100 g tuber). The changes of pectin and (hemi)cellulose contents are relative to the corresponding values of the wild-type potato variety. The wild-type background of the transgenic potato lines is shown as (a) Desiree, (b) Karnico, (c) Posmo, and (d) Kardal.

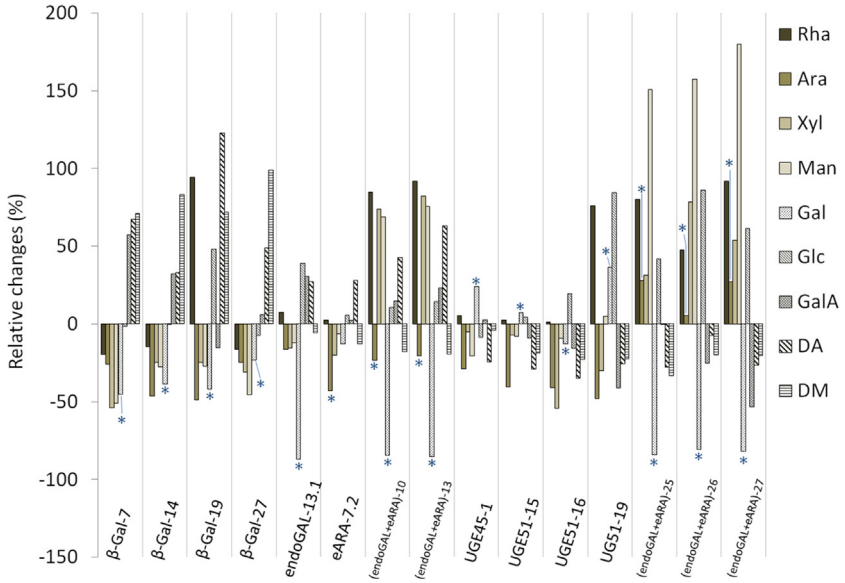

Fig. 4. Effect of transgenic modification of pectin side chains in β-Gal (7, 14, 19, and 27), endoGAL-13.1, eARA-7.2, endoGAL + eARA (10, 13, 25, 26, and 27), and UGE (45-1, 51-15, 51-16, 51-19) transgenic lines on monosaccharide content and acetylation (DA) and methyl-esterification (DM) of cell wall pectin. The changes of monosaccharide contents (mg/100 g tuber), DA, and DM are relative to the corresponding values of the wild-type potato variety. Asterisks denote the compounds that were affected owing to the targeted modification.

side chain-modifying transgenic lines targeting biosynthesis (e.g., UGE transgenic lines) showed a reduction of pectin esterification, whereas pectin side chain-modifying transgenic lines targeting polymer degradation (e.g., β-Gal) showed an increase in esters. The modified side chain length thus appeared to be accompanied with a change of the net charge of pectin, likely 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 line.

3.3. Modification of pectin acetylation

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

3.3.1. Targeted structures

The overexpression of PAE in potato tubers decreased the level of acetyl groups in CWM by 19% (Fig. 5). In comparison, Orfila et al. (2012) reported a 39% decrease in the acetyl groups present in

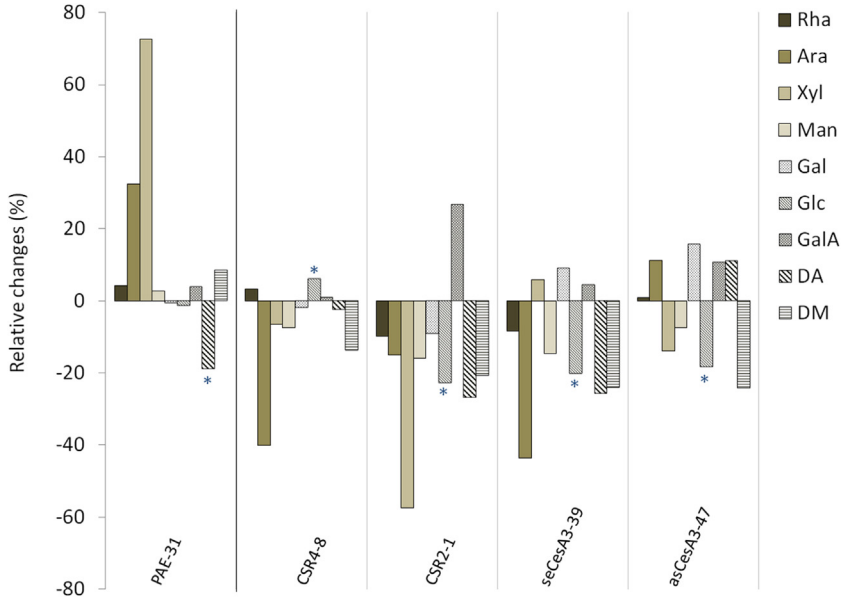

Fig. 5. Effect of transgenic modification of pectin esterification in PAE-31and cellulose level in CSR (4–8 and 2–1), CesA3-39, and asCesA3-47 transgenic lines on monosaccharide content, acetylation (DA), and methyl-esterification (DM) of cell wall pectin. The changes of monosaccharide content (mg/100 g tuber), DA, and DM are relative to the corresponding values of the wild-type potato variety. Asterisks denote the compounds that were affected owing to the targeted modification.

potato CWM. The difference in the decrease of acetyl groups might be due to the growing conditions used in each study.

3.3.2. 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 (Fig. 2C), or pectin and (hemi)cellulose content (Fig. 3C) when compared to wild-type varieties. In contrast, Ara and Xyl were markedly higher in the PAE transgenic line (Fig. 5).

3.4. Modification of cellulose level

Overexpression and silencing CesA genes, CesA or asCesA, has been used to increase or decrease the level of cellulose in potato tubers, respectively (Oomen, Tzitzikas, et al., 2004). CSR expressed in potato tubers was used to effect the specific down-regulation of the corresponding CesA genes (Oomen, Tzitzikas, et al., 2004).

3.4.1. Targeted structure

The reduction in Glc in the CSR2-1, CesA3-39, and asCesA3-47 transgenic lines (Fig. 5) resulted in an 18% to 24% decrease in (hemi)cellulose content (Fig. 3D). In previous work (Oomen, Tzitzikas, et al., 2004), 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.

3.4.2. Non-targeted structure

The entire constituent monosaccharide compositions of CSR, seCesA3, and asCesA3 are reported here for the first time (Supplementary Tables 1S and 2S). Higher HG:RG-I ratios in CSR2-1 (43%), CesA3-39 (19%), and asCesA3-47 (15%) were observed for this category (Fig. 2D). All transgenic lines showed a reduced DM of cell wall pectin (Fig. 5). In combination with the higher GalA level present in CWM, the non-esterified GalA located in the HG structural elements might provide a calcium-pectate complex to maintain the cell wall strength following cellulose loss. The reduced acetylation in all cellulose-targeting transgenic lines except asCesA3-47 might also improve the interaction of pectin with other cell wall polysaccharides providing a strong network within the cell wall architecture. Theoretically, the CSR lines should represent a lower cellulose level in comparison to wild type. The higher Glc content in CSR4-8 might due to the fact that the enzyme system was affected after transgenic modification, the unknown mechanisms of the extra Glc contents might influence the corresponding cell wall polysaccharides, which can provide a better strength during plant development.

3.5. 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, the potential side effects should always be considered when evaluating transgenic modifications of plants. The effects on the cell wall structures of each variety are summarized in Table 3. The evaluation provides an overview of each category (A–D) without considering the individual potato transgenic line.

3.5.1. Pectin backbone-modifying transgenic lines

Higher HG:RG-I ratios were found in all 11 transgenic lines (Table 3). Changing of the pectin backbone introduced side effects to the pectin side chains: 73% of the transgenic lines exhibited longer side chains length compared to that of wild-type varieties. About half of the pectin backbone-targeting transgenic lines showed either higher or lower acetylation levels of cell wall pectin compared to those of wild-type varieties, whereas the other half of the transgenic lines maintained similar levels of acetylation. Lower DM values were found for 6 transgenic lines. Most transgenic lines retained similar levels of pectin content compared to wild-type varieties. The transgenic lines that exhibited a modified pectin backbone also showed an increase in their (hemi)cellulose content (Table 3).

Table 3
Number of transgenic lines per category exhibiting changes in homogalacturonan:rhamnogalacturonan I (HG:RG-I), (Ara + Gal)/Rha, Gal/Rha, acetylation (DA), methyl-esterification (DM), pectin (Ara, Rha, Gal, and GalA) content, and (hemi)cellulose (Xyl, Man, and Glc) content. Values obtained from category (A) pectin backbone, (B) pectin side chain, (C) pectin esterification, and (D) cellulose level are given in comparison to that of 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 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: 11 transgenic lines in total.
[d] β-Gal, endoGAL, eARA, and (endoGAL + eARA) lines: 15 transgenic lines in total.
[e] PAE line: 1 transgenic line in total.
[f] CSR, CesA, and asCesa lines: 4 transgenic lines in total.

3.5.2. Pectin side chain-modifying transgenic lines

Over 70% of these lines showed a reduction in side chain length. Most also exhibited changes in the pectin backbone (low HG:RG-I ratios) when compared to wild-type varieties, which might be related to the presence of shorter side chains (Table 3). Based on the results of categories A and B, the (Ara + Gal):Rha, Gal:Rha, and HG:RG-I ratios 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 based on fresh tuber weight tended associate with a higher or similar hemi(cellulose) content to that of the wild-type. The change in DM might contribute to the strength of the cell wall structure.

3.5.3. Pectin esterification-modifying transgenic lines

In this category, only one transgenic line was available, which exhibited a lower degree of acetylation in the 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 or architecture.

3.5.4. Cellulose level-modifying transgenic lines

A decrease in (hemi)cellulose content was observed in this category. Half of the transgenic lines exhibited a longer pectin side length with simultaneously higher HG:RG-I ratio than wild-type. The cellulose level-transgenic lines retained similar amounts of pectin in the tuber as found for wild-type varieties.

3.5.5. Modified cell wall architecture

The precise mechanisms behind the modification of the level and structure of polysaccharides that were not targeted by the transgenic modification remain unclear. It is hypnotized that a targeted modification of potato cell wall polysaccharides has a direct effect on the cell wall architecture during tuber development, which might or might not result in a secondary compensatory set of changes in the cell wall polysaccharides. The modified cell wall polysaccharides might in turn alter the cell wall architecture, which might correlate to the absolute amount of CWM present. Moreover, modified pectin backbone structures in the Arabidopsis mutant quasimodo2 by reducing the HG blocks provide a higher flexibility of modified pectins during plant development (Ralet et al., 2008). Therefore, a modified pectin structure can provide different strength for cell wall architecture. Notably, a low yield of CWM was found for the pectin backbone-transgenic lines (category A, Supplementary Table 2S). In addition, it was observed that different levels of starch, stored as granules in the amyloplast of the potato tuber (Libessart et al., 1995), could be variously removed depending on the type of transgenic modification. This effect was most obvious for the transgenic lines targeting pectin side chains (category B, Supplementary Table 2S), where more residual starch was found in the CWM.

4. Conclusions

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.09.043.

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