TOWARDS UNRAVELLING THE BIOLOGICAL SIGNIFICANCE OF THE INDIVIDUAL COMPONENTS OF PECTIC HAIRY REGIONS IN PLANTS

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Abstract

Highly branched pectins, which are comprised of a rhamnogalacturonan (RG I) backbone carrying galactan and arabinan side-chains, are generally referred to as hairy regions. Even though composition of the hairy regions has been well established in many plants, their biological function is still unknown. Developmental studies have already shown distinct antibody labelling patterns for the different epitopes present on the hairy region, suggesting that they may have different functions. This review compares the results from the developmental studies together with those from mutagenized and genetically modified plants with compositional alterations to the hairy region. In particular, the specific degradation of hairy regions, by the introduction of fungal enzymes in potato, enables the assignment of a putative biological function to the constituent polymers of the hairy region. We hypothesize that the most important function of the galactan hairs is to regulate the pore size of the cell wall. The deposition of galactan may restrict the access of modifying enzymes to the wall. *In planta* fragmentation of the RG I backbone shows severe histological modifications in potato tuber tissue. This suggests that the RG I backbone has an important function for normal potato tuber cell division and tissue development.

1. Introduction

In potato (*Solanum tuberosum* L.), pectin is one of the major components of the cell wall, comprising about 56% (Figure 1) of the total cell wall material (Jarvis *et al.*, 1981; Vincken *et al.*, 2000). The pectic polysaccharides are suggested to form a matrix in the primary cell wall in which the load-bearing network of cellulose and cross-linking glycans is embedded (Carpita and Gibeaut, 1993). This pectic network is independent, but coextensive with the cellulose/xyloglucan network, and is suggested to structurally regulate the pore-size of the wall (McCann and Roberts, 1996).

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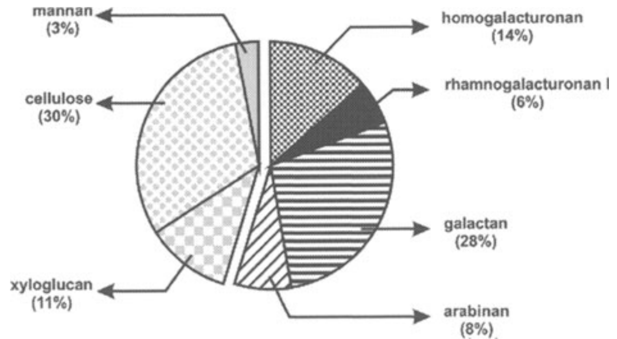

Figure 1.
Polysaccharide composition (% w/w) of the potato cell wall, representing all pectic polysaccharides, (except RG II) in black (56%), and other cell wall polysaccharides in gray {Vincken, Borkhardt, et al. 2000 271 /id}.

The primary structure of the individual pectic polysaccharides is well established. They comprise mainly homogalacturonan (HGA) and rhamnogalacturonan I (RG I). HGA (the smooth region) is composed of unbranched α-1→4-linked galacturonic acid (GalA) residues, which may be methyl-esterified and/or *O*-acetylated (Carpita and Gibeaut, 1993). RG I has a backbone composed of repeating α-(1→2)-L-rhamnose(Rha)-α-(1→4)-D-GalA disaccharide units. Also in the RG I backbone the GalA residues may be *O*-acetylated (Carpita and Gibeaut, 1993). Side-chains, mainly consisting of arabinan and/or galactan, may be attached to the RG I backbone at the *C*-4 position of the Rha residues (Carpita and Gibeaut, 1993; O'Neill *et al.*, 1990; Schols and Voragen, 1994). To date, there is little evidence that arabinan and galactan occur as independent polysaccharides in the cell wall. The macromolecular structure composed of RG I, galactan and arabinan is often referred to as hairy regions (Vries de *et al.*, 1981), with arabinan and galactan comprising the hairs.
However, much less information is available on the assembly of these polysaccharides into higher order structures and on their specific functions in the cell wall. The different pectic polysaccharides have been implicated in regulating cell expansion, organogenesis, textural changes during fruit ripening, and in serving as a source of oligosaccharins which can act as signalling molecules (Aldington and Fry, 1993; Darvill *et al.*, 1992; McCann and Roberts, 1994).
The physical properties of pectin make it suitable as a gelling agent, stabiliser or emulsifier in several food applications (Voragen *et al.*, 1995). Not all plant species are suitable sources of commercial pectin; currently mainly apple pomace and citrus peels, two by-products of food industrial processes, are used for pectin extraction. Commercially useful pectin should preferably have a high degree of methylation and a low proportion of hairy regions. The relatively high content of branched RG I, and low degree of methylation, make potato pectin a less suitable gelling agent for food applications (Ryden and Selvendran, 1990). The ability to modify the structure of potato tuber pectin *in planta* has the potential to increase the industrial applicability of potato fibre, which is a voluminous by-product of the potato starch industry (Chapple and Carpita, 1998; Thakur *et al.*, 1997; Vincken *et al.*, 2000).
Before it is possible to bio-engineer viable plants with pectin structures, which are improved for particular applications, it is necessary to understand the biosynthetic pathways for building and modifying the pectin. Further, it is important to know which pectin structures are present in various plants, plant tissues and cell types.

In this overview, we compare developmental studies, showing the naturally occuring variation in pectin structure, with studies on mutagenized and genetically modified plants. These approaches have helped to clarify the significance of the different hairy region structures for cell wall architecture/properties and developmental processes. Finally, this knowledge may enable the rational modification and future production of any desirable pectin structure *in planta.*

2. Pectin structure may determine tissue characteristics

Plants exhibit species-specific differences in their cell wall structure, together with compositional variations in the pectic polysaccharides, and their relative abundance. Determination of the monosaccharide compositions of different plant polymers provides useful structural information about pectic polysaccharides, but this approach fails to consider the importance of tissue- and cell-specific localisations of particular pectins.

The production of a series of antibodies recognizing different epitopes, each representing a part or particular structure of pectin, has clearly facilitated studies of developmental, tissue, and cell specific localization of the corresponding pectic structures (Knox, 1997; Willats *et al.*, 2000; Willats *et al.*, 2001a).

The monoclonal antibodies LM5 (recognizing (1→4)-β-D-galactan; (Jones *et al.*, 1997), and LM6 (recognizing (1→5)-α-L-arabinan; (Willats *et al.*, 1998), have been used extensively to study the presence and location of their respective epitopes in different plants and tissues at different developmental stages. These experiments have clearly indicated that hairy regions appear in a tightly regulated fashion with respect to cell wall localization, and also in relation to cell differentiation and cell proliferation (Bush *et al.*, 2000; Bush and McCann, 1999; Jones *et al.*, 1997; McCartney *et al.*, 2000; Orfila and Knox, 2000; Willats *et al.*, 1998; Willats *et al.*, 1999; Willats *et al.*, 2000; Willats *et al.*, 2001b).

In potato tubers, pectic epitopes are developmentally regulated during tuberisation and show also tissue-specific localisations in the mature tuber (Bush and McCann, 1999; (Bush *et al.*, 2001). An example of this are the labelling patterns of the LM5 and 2F4 (recognizing a calcium induced conformation of HGA; Liners *et al.*, 1994) antibodies as shown in Figure 2. The distributions of epitopes of galactan and HGA are complementary.

The LM5 and LM6 antibodies which both recognize RG I side-chains nevertheless show differences in labelling patterns. In stolons, the arabinan is enriched in the younger cells at the stolon tip (distal to the hook), while the galactan becomes more abundant in walls of older, elongated cells proximal to the stolon hook. In mature tubers, the arabinan epitopes are located throughout primary walls and middle lamellae of both cortical and perimedullary cell walls except at the expanded middle lamella at cell corners ((Bush *et al.*, 2001). Galactan is more abundantly localized in the primary walls of cortical and perimedullary tissues and in the primary cell wall it is mostly localized to the region close to the plasma membrane.

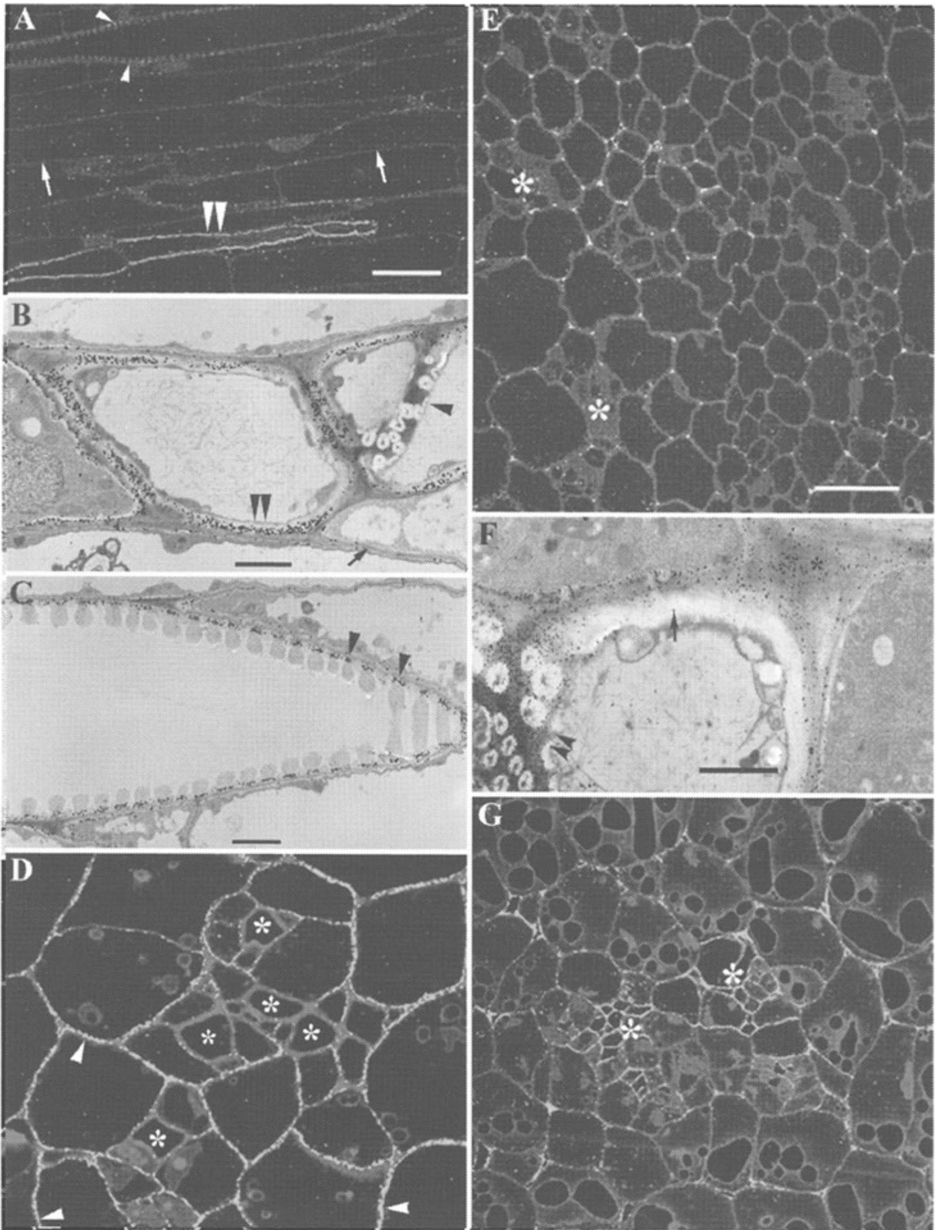

Figure 2. Sections of vascular tissue from elongating stolons (A-C, E-F) and 32mm mature tubers (D, G) immunogold labelled and silver-enhanced with monoclonal antibodies LM5 (A-D) and 2F4 (E-G) and then imaged by reflection confocal laser scanning microscopy (A, D-E, G) and transmission electron microscopy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

In vascular walls and at pit fields of parenchymal walls, the LM5 epitope is greatly reduced in abundance, whilst the abundance of the LM6 arabinan epitope is reduced to a lesser extent in vascular walls and is not altered at pit fields (Bush and McCann, 1999). These labelling studies clearly show that in potato some co-localisation of the galactan and arabinan epitopes occurs. Nevertheless, the distinct distribution of these epitopes indicates the existence of different populations of RG I bearing different hairs with the implication that each type may influence the developmental characteristics of the wall.

Galactans and arabinans of other plants also show differences in developmental and spatial localisation. (McCartney *et al.*, 2000) showed that, in pea cotyledon cell walls, both HGA and (1→5)-α-L-arabinan are continuously present, in contrast to a late developmental appearance of (1→4)-β-D-galactan. Further, the galactan was localised in the region of the cell wall close to the plasma membrane. This localisation of galactan is also found in other species (Jones *et al.*, 1997), and in most cases might be explained by deposition at a later stage of development (Bush and McCann, 1999; Vicré *et al.*, 1998). The developmental deposition of galactan in pea correlated with an increased firmness of the cotyledons (McCartney *et al.*, 2000). In tomato and kiwi, the loss of galactan is correlated with fruit softening and is expected to be an important trigger of additional cell wall changes associated with fruit ripening (Jones *et al.*, 1997; Redgwell *et al.*, 1997). The fact that these correlations were only found for the galactan and not for arabinan suggests that the different types of hairy regions have different functions in the cell wall.

It remains difficult to assign a specific characteristic of the wall or tissue to a particular pectic structure. The major reason for this is that during tissue development, other cell wall polymers will also be modified. The ability to produce plants with a specific, tailor-made, cell wall composition will be extremely useful to analyze the functions of individual cell wall polysaccharides further. In the following section we discuss different strategies to generate modified hairy region structures in plants. Even though little information is available concerning the biosynthesis of pectin, the generation of a number of mutants with a modified hairy region composition has shown the relevance of particular structures (Table 1). Together with this mutant strategy, a limited number of genetically modified plants have also been produced (Table 1). The possibility of targeted degradation of a particular hairy region domain by the expression of fungal pectinases has proven to be extremely useful and contributed significantly to revealing the biological function of cell wall polysaccharides.

Table 1. Plants with a modified RG I composition

Mutant / Transformant	Pectin modification	Phenotype	Mutated gene	Reference
Cnr	↓ de-esterified HGA, disrupted Ara deposition	disturbed fruit ripening, reduced cell-to-cell adhesion and non-swollen cell walls in the pericarp	?	Orfila *et al.* (2001)
emb30	abnormal localisation of the pectin	seeds unable to pass through normal embryogenesis, abnormal plants	similar to Sec7p, which functions in the secretory pathway	(Shevell *et al.*, 2000)
kor	↑ HGA ↓ RG I with galactan hairs	dwarfed, cellulose ↓	membrane-bound endo-1,4-β-glucanase	(His *et al.*, 2001)
mur5-6-7	↓ Ara[1]	not observed	?	(Reiter *et al.*, 1997)
mur4	Ara ↓ 50%	not observed	UDP-D-Xyl-4-epimerase	(Burget and Reiter, 1999)
mur8	↓ Rha	not observed	?	(Reiter *et al.*, 1997)
mur10	↓ Fuc and Xyl ↑ Ara	slow growth, dark-green leaves, low seed-set	?	(Reiter *et al.*, 1997)
mur11	↓ Rha, Fuc and Xyl ↑ Man	not observed	?	(Reiter *et al.*, 1997)
nolac-H14	abnormal localization of the pectin	has non-organogenic callus with loosely attached cells	?	Iwai *et al.* (2001)
* TBG4	no decrease in galactan	delayed and reduced fruit softening	not applicable	Brummell and Harpster (2001)
* UDP-Glc-4-epimerase	no galactan ↑ during growth on galactose medium	not observed	not applicable	Dörmann and Benning (1998)
** *e*GAL	Gal ↓	not observed	not applicable	Sørensen *et al.* (2000)
** *e*ARA[A]	?	no stolons, tubers, flowers and side shoots	not applicable	Skjøt *et al.* (2002)
** *e*ARA[B]	?	degenerate stolons, no tubers	not applicable	Skjøt *et al.* unpublished
** *e*GARA	Ara ↓	not observed	not applicable	Skjøt *et al.* (2002)
** *e*RGL	↓ Ara and Gal	disturbed histology of the potato tuber cortex and periderm tissue	not applicable	Oomen *et al.* (2002)

[1]It is not clear if this reduction is an effect of a decreased RG I bound arabinan or a decrease in AGPs
[A]Arabinanase expression under control of the granule bound starch synthase promoter
[B]Arabinanase expression under control of the patatinB33 promoter
no labelling = mutants
* = anti-sense expression of an endogenous gene
** = sense expression of a heterologous gene

3. The generation, isolation and characterisation of cell wall mutants

Most plants with a modified cell wall composition have been produced in *Arabidopsis thaliana* by a random mutagenesis approach, using chemicals, X-ray irradiation or T-DNA insertion (for an overview see (Fagard *et al.*, 2000). These mutants have been the starting point for the isolation and characterization of genes involved in the biosynthesis of cell wall polysaccharides.
Some of the cell wall mutants described so far have been isolated based on altered growth (His *et al.*, 2001). Identification of cell wall mutants is also possible by analysing the monosaccharide composition of isolated cell wall material, but this is a very laborious way to screen a large mutagenised population (Reiter *et al.*, 1997). New methods, with minimal sample preparation, have been developed to screen directly for modified cell wall polysaccharides. Such methods will facilitate the identification of new mutants. Fourier transform infrared (FTIR) microspectroscopy has proven to be a powerful tool for the screening of cell wall mutants (Chen *et al.*, 1998). Further, the various anti-pectin antibodies (Willats *et al.*, 2000) can be used for the identification of mutants with a modified cell wall composition (Willats *et al.*, 2001b).
A number of *Arabidopsis* mutants having modified hairy regions as a result of mutations in genes involved in pectic biosynthesis, degradation or deposition are shown in Table 1. The *Arabidopsis mur* mutants, as described by (Reiter *et al.*, 1997), were isolated by a screening based on monosaccharide composition analysis. Among these, *mur4, 5, 6* and *7* show a reduction of arabinose, which is due to a decrease in arabinan in either hairy regions or arabinogalactan-proteins (AGPs). The 50% reduction of arabinose in *mur*4 is too high to be accounted for solely by a reduction in AGP-arabinose. Further analysis of this mutant suggested a mutation in a membrane-bound UDP-D-xylose 4-epimerase, that blocks the conversion of UDP-D-xylose to UDP-L-arabinose (Burget and Reiter, 1999). The *mur8* mutant is reduced in rhamnose and the *mur10* has a modification in fucose, xylose and arabinose. The *mur11* mutants show modifications in rhamnose, fucose, xylose and mannose. Most of the *mur* mutants showed no visible phenotype; of the *mur* mutants listed in Table 1 only *mur10* showed a reduction in growth rate and vigour.
Not all mutants with modified pectin are likely to have a mutation in a gene directly involved in the biosynthesis or degradation of pectic polymers. An example is the *Arabidopsis emb30* mutant (Shevell *et al.*, 2000) with a mutation in a gene that is likely to be part of the secretory pathway. In these mutants the pectin shows an abnormal localisation, and the seeds are unable to develop into a normal plant which is most likely due to a lack of control of the polarity of cell divisions and expansions during embryogenesis. Another example is the *Nicotiana plumbaginifolia nolac-H14* mutant (Iwai *et al.*, 2001) which is characterized by having a *n*on-*o*rganogenic callus with *l*oosely *a*ttached *c*ells. In contrast to wild-type callus, the mutant, which can only be maintained as a callus line, does not stain with ruthenium red (recognizing pectin) in the middle lamella and cell walls, and the pectin seems to be relocated to the surface of the callus and the growth medium. Sugar composition analysis, comparing the *nolac-H14* with wild-type callus, shows relative decreases in arabinose, xylose, galactose, glucose and mannose in whole cell extracts and relative increases of these sugars and galactose in the culture medium. These changes are likely to be linked to an aberrant

deposition of pectin, although a modification in arabino-galactan proteins (AGPs) can not be excluded.
The *Cnr* ripening mutant of tomato also shows a disrupted deposition of several pectic polysaccharides (Orfila *et al.*, 2001). The mutant, for which the genetic basis has not yet been identified, has a pericarp with altered physical properties, including non-swollen cell walls and reduced intercellular adhesion (Thompson *et al.*, 1999). Even though no major differences were found by comparing the monosaccharide composition of the *Cnr* mutant and the wild type, antibody labelling clearly identified modifications in HGA and arabinan localization. A reduction of long de-esterified stretches of HGA possibly explains the reduced calcium-binding of the *Cnr* middle lamella, resulting in a reduced cell-to-cell adhesion. The additional disrupted deposition of (1→5)-α-arabinan did not correlate to the altered cell wall properties (Orfila *et al*,. 2001).
Modifications in pectin structure and localization can also be an indirect effect of modifications of other cell wall polymers. In the *Arabidopsis korrigan* mutant (His *et al.*, 2001), a mutation in a membrane-bound endo-1,4-β-glucanase resulted in a reduction of the cellulose content. This reduction in cellulose was accompanied with an increase in HGA and a decrease in RG I with galactan hairs, suggesting some compensatory mechanism for the cellulose-related reduction in wall strength. A similar result was observed in *Nicotiana benthamiana* after antisense inhibition of a cellulose synthase (*CesA*) gene (Burton *et al.*, 2000). These plants also showed that a decrease in cellulose content was accompanied with an increase of HGA with a decreased degree of esterification.
It is apparent from these studies that although the analysis of pectin mutants is a very useful approach to clarify the complexities of pectin biosynthesis, the generation of novel mutants remains a random process. In crop plants, the generation of mutants can be extremely difficult due to their polyploidy. An alternative here is genetic modification, which is possible in a large number of crop plants. Further, the down or up-regulation of an endogenous gene or the introduction of a heterologous gene provides a direct approach to specifically modify a known cell wall polysaccharide structure. Ideally, one would like to manipulate the biosynthetic machinery of the pectin, but unfortunately this approach is still limited since, to date, not many genes involved in wall polysaccharide biosynthesis have been cloned (Mohnen, 1999).

4. Genetic modification to generate plants with an altered cell wall composition

The biosynthetic and degradative pectic pathways can be targeted by genetic modification to generate cell walls with an altered pectin composition *in planta*. Genetic modification has already been used extensively to manipulate the processes causing fruit ripening and softening (reviewed by (Brummell and Harpster, 2001)). In these experiments, the expression of several genes (e.g. pectin methyl esterases and *endo*-polygalacturonases) responsible for modification of the pectin structure were down-regulated. These experiments are not further discussed here since the activity of these enzymes is mostly directed to HGA and not to the hairy regions. An important modification of the hairy regions during fruit ripening is the decline in pectic galactan early in the ripening process (reviewed in Brummell and Harpster, 2001). Suppression of the TBG4 gene, encoding a β-galactosidase activity (Smith and Gross, 2000) in

tomato, showed a reduced fruit softening (Brummell and Harpster, 2001). This suggested that RG I galactan chains contribute to maintaining the firmness of the fruit, and that their degradation by endogenous enzymes is one of the changes leading to fruit softening during ripening.

An alternative direct approach to obtain a modified cell wall composition in plants is by manipulation of the biosynthetic machinery. One example of such a biosynthetic gene is the UDP-Glc-4-epimerase, which was used for sense and antisense expression in *Arabidopsis* (Dörmann and Benning, 1998). The enzyme, which catalyzes the reversible epimerization of UDP-galactose to UDP-glucose, is important in maintaining the pool of UDP-galactose that is the building block for the galactan hairs. The sense and antisense expression resulted in 3-fold increases and 90% reduction of the corresponding enzyme activity, respectively. Nevertheless, this did not result in a modified composition of the pectin during normal growth of the plants. Growth of the *Arabidopsis* plantlets on agar containing galactose, increased the amount of cell wall bound galactose in both wild type as well as in antisense plants with a decreased UDP-Glc-4-epimerase activity. In plants with an increased epimerase activity this effect was not found. This suggests that the UDP-Glc-4-epimerase is indeed important for maintaining the UDP-galactose pool and indirectly influences the amount of cell wall bound galactose. Nevertheless, in these transformants, enzyme levels as low as 10% of wild type are still sufficient to maintain normal UDP-galactose levels.

5. The introduction of pectin-degrading enzymes in potato

5.1. Fungal enzymes with potential to degrade hairy regions

Many enzymes degrading different pectic polymers have been described, and even though some of these enzymes are from plants (Fry, 1995), the best-characterised enzymes originate from fungi and bacteria (for an overview see Prade *et al.*, 1999).

Focussing on the removal of the branched part of the pectin, five hairy region-modifying enzymes, which can be divided in two categories, have been introduced in potato so far (Oomen *et al.*, 2001; Skjøt *et al.*, 2001a; Sørensen *et al.*, 2000). The first category encompasses an *endo*-galactanase and an *endo*-arabinanase, which are referred to as 'shaving enzymes'. These remove the galactan and arabinan side-chains of the RG I leaving the backbone polymer intact. The *endo*-galactanase (*e*GAL) from *Aspergillus aculeatus* is able to degrade (unsubstituted) 1→4-β-D-galactan to galactose and galactobiose (Christgau *et al.*, 2001; Lahaye *et al.*, 1991; Vis van de *et al.*, 1991). The *endo*-arabinanase (*e*ARA) also from *Aspergillus aculeatus* hydrolyzes α-1→5-L-arabinan and is specific for α-1→5-L-arabinofuranosidic linkages (Skjøt *et al.*, 2001b). The second category includes the 'breaking enzymes' which degrade the RG I backbone and liberate the hairs attached to small backbone fragments. The rhamnogalacturonan hydrolase (*e*RGH) (Mutter *et al.*, 1996; Schols *et al.*, 1990) cleaves the backbone by hydrolysis at the non-reducing side of a rhamnosyl residue. In contrast, rhamnogalacturonan lyase (*e*RGL) cleaves the RG I backbone at the reducing side of the rhamnose. *In vitro* experiments have shown that de-acetylation of an RG I preparation by addition of a rhamnogalacturonan acetyl esterase (RGAE) enhances degradation by both *e*RGL and *e*RGH (Kauppinen *et al.*, 1995; Kofod *et al.*, 1994;

Schols *et al.*, 1990). This synergy can also be used *in planta* by expressing the RGAE together with the *e*RGL or *e*RGH.

5.2 INTRODUCTION OF HAIRY REGION DEGRADING ENZYMES IN POTATO

Modifications to cell walls could potentially have adverse effects on plants. These secondary effects might be limited by using tissue-specific promoters and specific targeting signals. In the majority of the experiments described below the granule-bound starch synthase (GBSS) promoter (Visser *et al.*, 1991) was used to obtain preferential expression of the genes in the potato tubers, and thus limit the effect on other parts of the plant. Since signal peptides are very similar among eukaryotes (Nielsen *et al.*, 1997), the endogenous targeting signals in these fungal enzymes were sufficient to permit targeting of the protein to the apoplast. The transformation efficiency with the *e*ARA and *e*GAL genes (Skjøt *et al.*, 2001a; Sørensen *et al.*, 2000) showed a significant decrease compared with the transformation of empty vector constructs, even though no significant differences were found for the other genes. The introduction of the genes did not have a large effect on the potato plants, which showed a clear expression of the transgene in the potato tubers.

*e*GAL, *e*ARA and *e*RGL enzyme activities were demonstrated in tissue extracts of the corresponding transformants and were not present in wild type tubers. No RG I-degrading activity was found in transformants expressing the *e*RGH or *e*RGH + *e*RGAE gene combination (unpublished results). Western and dot blot analysis using *e*RGH and *e*RGAE specific antibodies could not detect these two proteins in whole tuber extracts. Fourier transform infrared (FTIR) microspectroscopy and antibody labelling studies showed no alterations in cell wall composition in the *e*RGH or *e*RGH + *e*RGAE transformants. RNA was expressed for both *e*RGH and *e*RGAE, this together with the absence of the proteins suggests a problem during translation or post-translational modification of these two enzymes.

Both the *e*RGL and *e*ARA transformants showed phenotypic differences, compared to the wild type plants. The *e*RGL tranformants showed only phenotypic changes in the tubers (Oomen *et al.*, 2001), which had a more wrinkled morphology compared with the wild type tubers (figure 3).

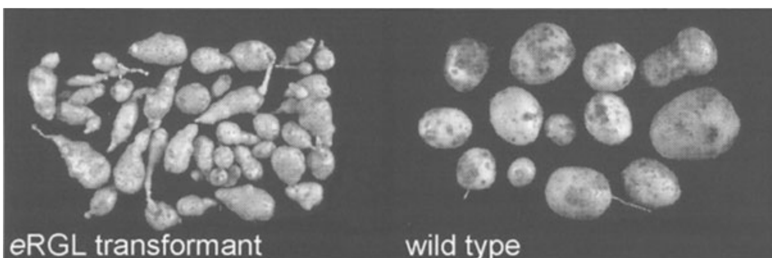

Figure 3. A selection of wild type potato tubers and tubers produced by a transformant expressing the *e*RGL gene.

The *e*ARA transformants showed a more severe phenotype. Plants expressing the arabinanase under control of the GBSS promoter produced no side shoots, flowers, stolons and tubers (Skjøt *et al.*, 2001a). Replacement of the GBSS promoter with the patatin B33 promoter (Rocha-Sosa et al., 1989) yielded plants with normal aerial parts (Skjøt et al. unpublished results). However, these plants developed degenerated stolons (Figure 4). Scanning electron microscopy (SEM) revealed major morphological differences between transgenic and wild type stolons (Figure 4). Wild type stolons viewed by SEM are long, slender diageotropic stems with a hooked tip bearing an apical meristem surrounded by leaf primordia (Fig. 4A). As the stolon tip elongates, meristematic nodes are left behind between the angle of the leaf primordia and the stolon. Leaf primordia bear trichomes on both surfaces (Fig. 4 A and B) and numerous stomata (Fig. 4C). In contrast, transgenic stolons are multi-branched stems with many secondary stolons (Fig. 4D) that develop from the leaf primordial nodes along the length of the primary stolon. Transgenic leaf primordia develop normally, but lack the numerous trichomes seen in the wild type (Fig. 4E), whilst the stomata are characteristically raised above the irregular epidermal surface (Fig. 4F).

Due to the inhibition of normal stolon and tuber development in the arabinanase-expressing potato plants, we have not been able to show, by using conventional biochemical techniques and immunogold labeling with the arabinan specific antibody LM6, that apoplastic expression of the endo-arabinanase results in a changed RGI sugar composition or epitope abundance. The carbohydrate moieties of cell wall associated proteoglycans may also be hypothetical substrates for the arabinanase when expressed to the apoplastic space. However, to the best of our knowledge, all arabinosylated hydroxyproline rich glycoproteins (HRGPs) analyzed to date except one, only contain a single terminal 1,5 linked arabinosyl residue which is not a substrate for the *A.aculeatus* endo-arabinanase (Skjøt *et al.*, 2002).

The sole described exception is a proteoglycan that has been isolated from cultured *Acacia senegal* cells. This arabinogalactan protein (AGP) carries side-chains consisting of α-1,5-linked arabinosyl groups (Mollard and Joseleau, 1994). The HRGPs are a large and heterogeneous protein family (Sommer-Knudsen *et al.*, 1998) and the finding that the *Acacia senegal* AGP carries arabinan side chains consisting of 1,5-linked arabinose does raise the possibility that non-hairy region arabinans may serve as substrate for the endo-arabinanase.

The phenotype of the *e*ARA plants clearly exemplify that a post-depositional degradation approach is not universally applicable and that other approaches must be used for *in vivo* enzymatic modification of α-1,5-linked arabinans.

Interestingly, a pre-deposition strategy where the arabinanase was targeted to the site of pectin biosynthesis, the Golgi apparatus, resulted in transformants (*eG*ARA) with a phenotype indistinguishable from WT and results in development of normal tubers (Skjøt *et al.*, 2001a). Expression of this chimeric enzyme engineered to be Golgi-membrane anchored resulted in a 69 % reduction of RG I-associated arabinosylgroups. This suggests that the severe phenotype of the apoplastic *e*ARA transformants is not an effect of the hairy regions lacking arabinan side chains. It is possible that in the *e*ARA plants normal tuber organogenesis is disturbed by secondary effects of released degradation products or by the consequences of enzymatic hydrolysis of non-hairy region associated arabinans.

Clearly, these results suggest that cell wall associated galactans and arabinans in the

form of side chains of the hairy regions or as polysaccharide substituents on e.g. AGPs play different roles during plant development.

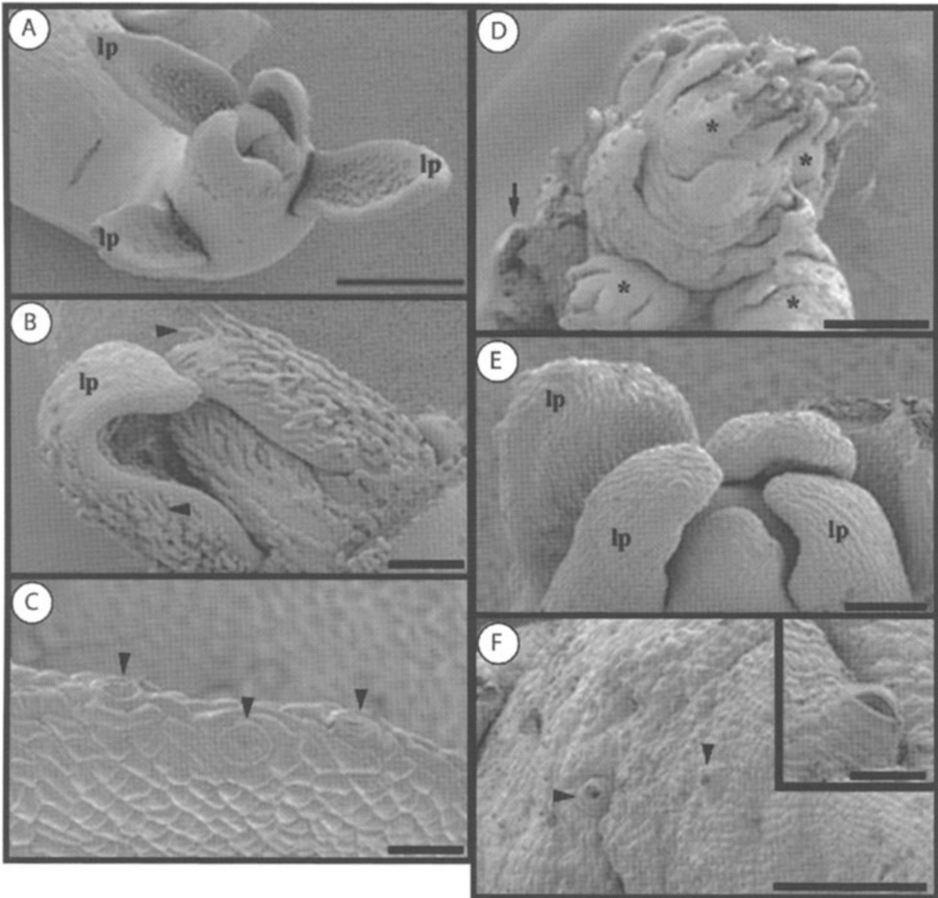

Figure 4. Scanning electron micrographs of stolons from wild type (A-C) and *e*ARA transformant with the arabinanase under control of the patatin B33 promoter (D-F). A: The tip of the wild type stolon bends through 90° to give a characteristic hook that bears leaf primordia (lp). In B the outer leaf primordia have been removed to reveal the numerous trichomes (arrowheads) that decorate both surfaces of the inner leaf primordia. C: Wild type stomata are normal in appearance (arrowheads). D: A short, brush-like arabinanase-expressing stolon bearing numerous secondary stolons (asterisks), the arrow indicates where the primary stolon was attached to the main stem of the plant. E: Transgenic leaf primordia lack the trichomes seen in the wild type plant (compare with A and B). F: The epidermal surface is very irregular and the stomata are abnormally raised (see insert). Scale bars are 1mm, D; 0.5mm, A; 200μm, B, F; 100μm, E and 50μm, C, inset in F.

5.3 EFFECT ON THE CELL WALL COMPOSITION

In addition to the predicted enzyme activities in the transgenic plants, both biochemical and microscopic analysis showed that the enzymes were able to modify the hairy region composition *in planta*.
The differences in the sugar composition of cell wall material isolated from the different transformants (Oomen *et al.*, 2001; Skjøt *et al.*, 2001a; Sørensen *et al.*, 2000) are summarized in Table 2. Both the *e*GAL and Golgi-targeted *eG*ARA transformants showed a reduction in galactose and arabinose respectively to a level corresponding to about 30% of the wild type. These reductions did not lead to obvious alterations in the other sugar residues, which suggests that these modifications are not compensated. Microscopic analysis, using the LM5 and LM6 antibodies respectively recognizing (1→4)-β-D-galactan (Jones *et al.*, 1997) and (1→5)-α-L-arabinan (Willats *et al.*, 1998), confirmed these biochemical observations. The *e*GAL and *eG*ARA transformants showed a reduced labelling with LM5 and LM6 respectively. No phenotypic changes were found in these transformants.

Table 2. Modifications in the sugar composition of the potato tubers from the *e*RGL, *e*ARA and *e*GAL transformants in comparison with tubers from wild type plants

	*e*RGL	*eG*ARA	*e*GAL
Rhamnose	–	≈	≈
Arabinose	–	–	≈
Galactose	–	≈	–
Mannose	+	not determined	≈
Xylose	+	≈	≈
Glucose	≈	≈	≈
Uronic acid	+	≈	≈

+ and – represent, respectively, increases and decreases of the particular sugar residue in comparison to wild type levels
≈ indicates no major changes in the particular sugar residue

In the *e*RGL transformants, where the RG I is degraded as opposed to 'shaved' as with the *e*GAL and *e*ARA, a greater impact on the potato tuber phenotype was observed. Sugar composition analysis showed a reduction of both galactose and arabinose to levels corresponding to respectively 20 and 57% of the wild type, this was accompanied by relative increases in mannose, xylose and uronic acid. Antibody labelling studies with LM5 and LM6 showed that both epitopes were reduced in abundance in walls, indicating that the side-chains were released from the cell wall by a fragmentation of the RG I backbone. Together with the reduced antibody labelling there was also a relocation of the LM5 galactan epitopes towards the middle lamella at cell corners and intercellular spaces. These relocations and reductions suggest that the RG I side chains are not cross-linked to other wall polymers, since the *e*RGL can only degrade the RG I backbone (Kofod *et al.*, 1994). The relocation of the hairy region components in the cell wall is only observed in the *e*RGL transformants and not in the *e*GAL and *eG*ARA plants.

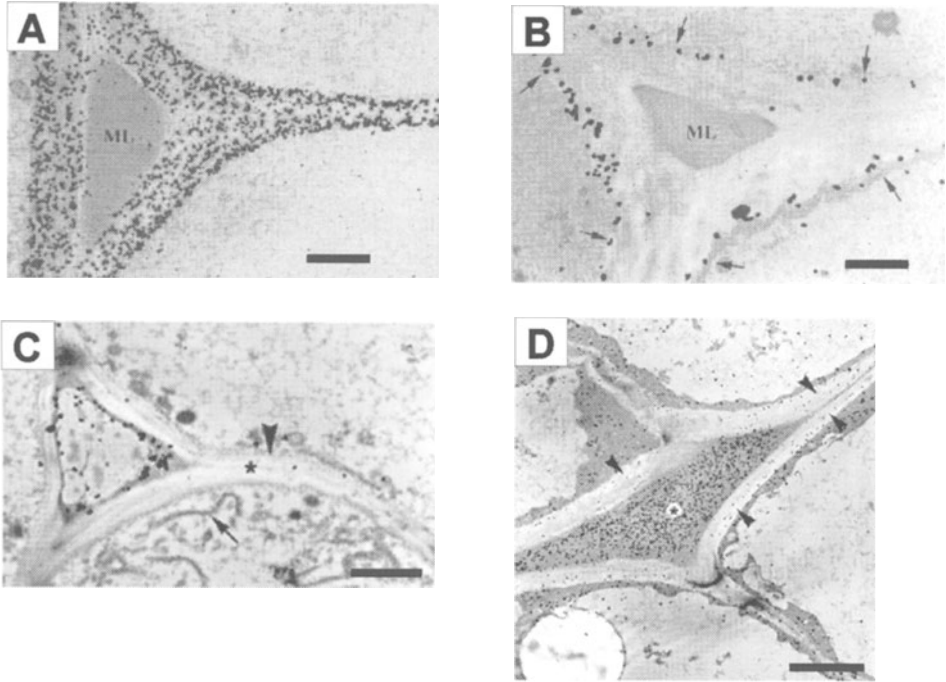

Figure 5. Transmission electron micrographs of cell walls from wild type (A), *e*GAL (B) and *e*RGL (C-D) expressing tubers immuno-labelled with LM5. ML (in A and B) and asterisk (in D) indicate the expanded middle lamella. Asterisk in C indicates the middle lamella. Arrows and arrowheads in B, C and D indicate remaining LM5 labelling. Scale bars represent 2 µm (A, B and C) and 4 µm (D).

Figure 5 shows the LM5 labelling in transmission electron micrographs for the *e*GAL, *e*RGL and wild type potato tubers. In wild type tubers, the LM5 epitopes are located in the primary cell wall and are excluded from the middle lamella and cell corners. The *e*GAL tubers show a reduction of LM5 epitopes and some remaining epitopes close to the plasma membrane. In the *e*RGL tubers, most of the epitope is present in the middle lamella at cell corners and there is a reduced epitope abundance in the primary wall. The remaining LM5 epitopes in the *e*GAL are likely to be located closer to the RG I backbone of the hairy region due to the (partial) degradation of the galactan hairs. The localization of these residual epitopes close to the plasma membrane suggests that (part of) the RG I (with enough remaining galactan to be still recognized by LM5) is located there. This RG I could either be anchored at this location or could represent newly deposited RG I with galactan hairs. However, it would be expected that newly deposited intact RG I would lie very close to the plasma membrane and that as it became incorporated into the wall, the *e*RGL would degrade it. As we did not see LM5 or LM6 labelling very close to the plasma membrane in the *e*RGL transformants, this would argue against the residual epitopes belonging to newly deposited RG I. We interpret the residual epitopes as belonging to a population of RG I backbones anchored close to the plasma membrane. The release of both galactan and arabinan after fragmentation of the RG I backbone confirms that they are only attached to the RG I

backbone. This is consistent with the results of (Foster *et al.*, 1998) and (Fenwick *et al.*, 1999) who showed that galactan side-chains in onion parenchyma walls are attached to a less mobile molecule at one end but are otherwise very mobile.
Both LM5 and LM6 are indirect antibody markers to detect RG I since they only recognize the respective side-chains and not the backbone. An antibody recognizing the RG I backbone structure could indicate if the relocated fragments contain RG I backbones or if RG I structures are still left in the cell wall. The mAb CCRC-M2 (Puhlmann *et al.*, 1994) does not cross-react with potato (Bush and McCann, 1999).
The *e*RGL potato tubers have a modified histology (Oomen *et al.*, 2001). In contrast to wild type, cell layers in the periderm and cortex tissues of transformants are severely disorganised and the number and size of cortical intercellular spaces is increased.
The comparison of the *e*GAL, *eG*ARA and *e*RGL transformants shows the importance of the complete hairy region structures, or the RG I backbone in particular, compared to its individual galactan and arabinan side-chains. The histological and morphological changes in the *e*RGL transformants suggest an important role of the RG I backbone for the integrity and function of the wall, at least in potato tubers.

6. A possible biological function for RG I and the hairs?

The expression of fungal cell wall degrading enzymes in plants was a useful approach to study pectic polysaccharide function. The specific deletion of parts of the hairy region enabled us to assign a putative function to the galactan hairs and the RG I backbone. Even though the *e*RGH and *e*RGAE were not successfully translated into a functional protein, the *e*GAL, *eG*ARA and *e*RGL enzymes modified pectin structure *in planta*. Nevertheless, tissue specific expression and subcellular localization were important determinants to confine the consequences of these modifications (Skjøt *et al.*, 2001a). Future use of constructs where the expression of the hairy region modifying enzymes is under control of inducible promoters may be beneficial.
The *e*GAL transformants (Sørensen *et al.*, 2000) are a useful tool to study the function of the RG I galactan hairs. It has been shown that galactan is present mostly in relatively older cells of a number of plant tissues (Bush *et al.*, 2001; Vicré *et al.*, 1998; Willats *et al.*, 1999). Some of these developmental changes, showing either addition (McCartney *et al.*, 2000) or degradation (Jones *et al.*, 1997; Redgwell *et al.*, 1997) of galactan, suggest that galactan is positively correlated with firmness of the plant tissues. However, in the *e*GAL transformants, with a reduction of RG I galactan hairs, there were no indications of altered tuber firmness, development or morphology. Nevertheless, the suppression of galactan degradation in tomato fruit, by antisense expression of a β-galactosidase/exo-galactanase (Brummell and Harpster, 2001), reduced fruit softening, but did not stop this process.
Combination of the results from the *e*GAL potatoes with the observations of pea and fruit ripening suggests that removal of the galactan chains does not directly reduce the strength of the cell wall. It is likely that the loss of galactan chains enables other enzymes to access regions of the cell wall to modify other cell wall components. This is in line with the observations by (Foster *et al.*, 1998) and (Fenwick *et al.*, 1999), who suggested that galactan hairs might be important for decreasing the pore size of cell walls and hence play a role in wall porosity. Nevertheless, the exact mechanism by

which galactan deposition can decrease the pore size is unclear. In contrast to RG II, which is suggested to actively determine the pore size in the cell wall by forming a pectic network with borate ester cross-linking (Fleischer *et al.*, 1999), the galactan is probably only decreasing the pore size by functioning as a filling material.
The fact that *e*GAL expression in potato tubers had no detectable effect on tuber development and morphology (Sørensen *et al.*, 2000), may be due to an absence of endogenous cell wall modifying enzymes in the tubers when the *e*GAL was expressed. This may explain why the loss of RG I galactan hairs did not have a similar impact to galactan degradation in fruit ripening processes. It seems that galactan degradation has only an impact when other wall polysaccharidases are expressed concomitantly. This is in line with the observation that a down regulated TBG4 expression always gives a reduced galactan degradation but results only in a delayed softening when the down regulation takes place early in the ripening process (Brummell and Harpster, 2001). Since potato tubers, directly after their formation, enter a dormant period, these organs are not likely to show many structural and or textural changes as a result of galactan degradation.
No particular function has been suggested to date for the RG I arabinan hairs, even though they are developmentally and spatially regulated (Bush *et al.*, 2001). The severe phenotype of the apoplastic *e*ARA transformants is likely to be caused by secondary effects or possibly by enzymatic hydrolysis of non-hairy region associated arabinans and not purely by a removal of arabinan from RG I. This is supported by the normal phenotype of transformants in which the *eG*ARA protein was targeted to the Golgi (Skjøt *et al.*, 2001a) and the arabinan content in the wall was successfully reduced. These latter *eG*ARA transformants are comparable with the *Arabidopsis mur4* mutant (Burget and Reiter, 1999) which also shows a reduction in L-arabinose (respectively 69 and 50%). These plants do not show any phenotypic defect and the reduction in L-arabinose does not seem to be compensated for by another specific monosaccharide. This suggests that a reduced level of RG I bound arabinan (at these environmental and physiological conditions) does not interfere with normal plant development although the extended phenotypes of the plants remain to be described.
The altered histology and morphology of potato tubers expressing the *e*RGL suggests an important function of the RG I backbone structure, since the removal of only the galactan and arabinan hairs did not result in cytological differences. The altered tuber morphology in the *e*RGL plants, caused by changes in the periderm and cortex, are probably the result of random divisions and abnormal expansions in these tissues. These results suggest that the RG I is necessary for normal periderm development even though the underlying reason is not yet clear. A similar effect was found in the *Arabidopsis emb30* mutants (Shevell et al., 2000). These mutants show a localization of pectic polysaccharides at cell corners and interstitial spaces together with the normal wild type localization in the cell wall. The authors hypothesize that the abnormal localization, which is likely to be caused by a failure in the deposition of the pectic polysaccharides, might interfere with normal cell division and disturb the cellular polarity. It is not known that the mutation in *emb30* directly affects the hairy region, but it cannot be excluded that the abnormalities found in the *e*RGL transformants may be the result of a similar mechanism.
Primary analysis of the first generation of genetically modified plants with an altered RG I composition was very useful in assigning a putative biological function to the

galactan hairs and the RG I backbone. The deposition of RG I with galactan hairs is likely to alter access of wall-modifying enzymes to the cell wall by decreasing the pore size. RG I backbones in the wall appear to be necessary for normal cell division and tissue development in potato tuber periderm and cortical tissues. Nevertheless, much more research on these, and future plants, is necessary. Apart from the generation of an antibody recognizing the potato RG I backbone, the generation of double transformants containing both the *e*GAL as well as the *eG*ARA will help to confirm if indeed the fragmentation of the RG I backbone structure (as in the *e*RGL transformants) is causing the altered potato tuber morphology. Analysis of the *e*GAL and *eG*ARA tranformants under normal growth conditions has so far not revealed any far-reaching implications for wall architecture or plant development. It should be noted here that the potato tubers were studied during the dormancy stage, in which developmental and biochemical processes are at a very low level. It is likely that e.g. an increase in the pore size of the cell walls in the *e*GAL transformants, may have an effect at other developmental stages, for instance, sprouting of the tubers. Studies of the extended phenotypes of these plants during different developmental stages and at different environmental and physiological conditions might reveal differences between the various transformants and wild type plants. It is expected that the use of these transgenic plants in developmental studies will deliver an important contribution and may result in a detailed description of the biological functions of the hairy regions.

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