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Cell wall as a barrier for protein extraction from tomato leaves: A biochemical study

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A R T I C L E I N F O

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A B S T R A C T

Solanum lycopersicum (Tomato) leaves and stems are considered waste. Valorization of this waste can be achieved by for example the extraction of proteins. This prospect is promising but currently not feasible, since protein extraction yields from tomato leaves are low, amongst other due to the (physical) barrier formed by the plant cell walls. However, the molecular aspects of the relationship between cell wall properties and protein extractability from tomato leaves are currently not clear and thus objective of this study. To fill this knowledge gap the biochemical composition of plant cell walls was measured and related to protein extraction yields at different plant ages, leaf positions, and across different tomato accessions, including two *Solanum lycopersicum* cultivars and the wildtype species *S. pimpinellifolium* and *S. pennellii*. For all genotypes, protein extraction yields from tomato leaves were the highest in young tissues, with a decreasing trend towards older plant material. This decrease of protein extraction yield was accompanied by a significant increase of arabinose and galacturonic acid content and a decrease of galactose content in the cell walls of old-vs-young tissues. This resulted in strong negative correlations between protein extraction yield and the content of arabinose and galacturonic acid in the cell wall, and a positive correlation between the content of galactose and protein extraction yield. Overall, these results point to the importance of the pectin network on protein extractability, making pectin a potential breeding target for enhancing protein extractability from tomato leaves.

1. Introduction

Tomato (*Solanum lycopersicum*) is a major crop, cultivated on approximately 420,000 ha of agricultural land in Europe (FAOSTAT, 2023). While tomato fruits are widely commercially utilized, the leaves often represent crop waste, as they hardly find an industrial use. The latter might change in the context of a circular economy, which strives for the utilization of all plant components. In this context, recent research by Arab et al. (2019) demonstrated that the extraction of valuable phytochemicals and proteins from tomato leaves can increase the commercial value of the tomato crop.

Tomato leaf proteins offer a great economic potential for valorization due to their high abundance, as they constitute up to 28% of the dry matter content of tomato leaves (Abo Bakr, 1982; Yu et al., 2023). The majority of the proteins in leaves is associated with photosynthesis, corresponding to up to 80% of the total protein content within chloroplasts (Fiorentini and Galoppini, 1983). Approximately 50% of chloroplast proteins are ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39); the most abundant protein on earth (Ellis, 1979). Therefore, RuBisCO and all other photosynthesis-related proteins in the chloroplasts have become the primary target for protein extraction. However, extracting proteins from chloroplasts presents several challenges, of which the disruption of leaf and cell structures, particularly the cell walls, is often considered as the major physical barrier (Phong et al., 2018; Safi et al., 2013; Tamayo Tenorio et al., 2018). Plant cell walls provide mechanical strength and protection against various (a)biotic stresses (Le Gall et al., 2015; Lima et al., 2013; Solecka et al., 2008; Ishida and Noutoshi, 2022). These functions are conferred by the matrix of polysaccharides and polyphenols that constitute cell walls themselves and whose physical structure and chemical composition have evolved to impede easy cell wall deconstruction (Sarkar et al., 2009). Therefore, cell walls represent a potential

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challenge when reaching for high protein extraction yields (Safi et al., 2013; Tamayo Tenorio et al., 2018; Rommi et al., 2014; Bals et al., 2009; Sari et al., 2015; Zhang et al., 2015).

The impact of cell walls on the extraction yields of different components from various plant tissues has been studied in some other crops than tomato. For example, the extraction of tannins from grape wine leaves was constrained by pectic polymers and extensins (Boulet et al., 2023), while the quantity of anthocyanins was limited by the amount of galactose, rhamnose and xylose as well as by the degree of pectin acetylation and methylation (Ortega-Regules et al., 2006). Similarly, pectins were found to hamper protein extraction from sugar beet leaves, since treatment of leaf tissues with pectin degrading enzymes (pectinases) increased protein extraction yield by 2.2-fold (Akyuz and Ersus, 2021). Finally, in ryegrass and clover, protein extraction yield was improved by the use of carbohydrases and proteases (Dotsenko and Lange, 2017).

To understand which components of the cell walls interfere with protein extraction, it is essential to understand the biochemical structure of plant cell walls. Generally, plant cell walls consist of primary and secondary cell walls. Primary cell walls are synthesized during cell expansion in all plant cells, and are formed by a matrix of cellulose, hemicellulose and pectins. Conversely, secondary cell walls are deposited at the inner part of the primary cell walls once the cell growth ceases, and differ from primary cell walls as they contain negligible amounts of pectins and considerable amounts of lignin (Buchanan et al., 2015; Taiz et al., 2015; Liepman et al., 2010; Kumar et al., 2016). Moreover, secondary cell walls are typically deposited in vascular tissues, while they are nearly absent in leaves, which are largely formed by parenchyma cells (Akin and Burdick, 1975). With regard to the relative amounts of the different cell wall components, data on tomato leaf cell walls are scarce, thus information regarding the constituents of dicot plant cell walls can offer a comprehensive perspective. Generally, in dicot plants, pectin is the most abundant cell wall component, followed by cellulose and hemicellulose (Rose, 2003; Zablackis et al., 1995; Vogel, 2008). Pectin comprises homogalacturonan and the complex polymers rhamnogalacturonan I and II. The primary constituent of pectin is galacturonic acid, accompanied with significant amounts of arabinose and galactose (Anderson, 2019; Mohnen, 2008). Cellulose, responsible for the core mechanical strength of the cell wall, is formed by β-1,4 linked glucose chains (Taylor, 2008), while hemicellulose consists of xylose, glucose and mannan (Vogel, 2008; Ebringerová et al., 2005). Subsequent to the cell wall synthesis, dynamic remodeling and restructuring in response to cellular growth and various environmental stresses occurs. Notably, one aspect of this process involves a decrease in esterification within the pectin network (Anderson, 2019). To conclude, it is relevant to highlight that the composition and modification of cell walls changes with plant age, which can also affect protein extraction, particularly in tomato leaves (Yu et al., 2023).

The aim of this study is to understand if and how plant cell walls affect the protein extraction yield from tomato leaves. To achieve this goal, we determined protein extraction yield and biochemically analyzed plant cell walls across four tomato genotypes from three different tomato species: two *Solanum lycopersicum* cultivars*,* one *Solanum pimpinellifolium*, and one *Solanum pennellii*. On top, the aging effect was also considered by including five timepoints across different developmental stages and three leaf positions in our analyses. This approach allowed us to establish correlations between the protein extraction yield from tomato leaves and the leaves cell wall content and biochemical composition. Finally, the obtained findings are discussed in relation to both their fundamental impact and the identification of molecular breeding targets for enhancing protein extractability.

2. Material and methods

2.1. Plant material

Four tomato genotypes were used in this research, including two cultivars of *Solanum lycopersicum,* cultivar Moneymaker and cultivar Gardeners Delight, and two related wild tomato species – *S. pimpinellifolium* (accession G1.1596) and *S. pennellii* (accession LA716). The selection of four genotypes was made, to identify the robustness of our findings. The tomato seeds were sown in soil in early September 2021, transferred to rockwool blocks 14 days after sowing (DAS) and placed on stonewool slabs at 46 DAS. The plants were grown in the greenhouse, under the conditions specified in the Supplementary Fig. S 1. Plants were harvested at different timepoints, determined by the developmental stages of Moneymaker (vegetative, flowering, fruit forming, mature fruits stage I and II), as this genotype is the most commonly studied genotype in tomato research (Table 1)(Reiter, 1998). For each time point, three individual biological replicates were harvested. Given the small size of the plants at the vegetative state (37 DAS), three or four plants per genotype were pooled to gain a sufficient quantity of leaves for the following analysis. Similarly, at the flowering stage (50 DAS), two (*S. Pimpinellifolium*) and three (*S. Pennellii*) plants per replicate were pooled, while for Moneymaker and Gardeners Delight one plant yielded sufficient quantity (>20g) of leaf material to perform all analyses. From the fruit forming stage (77 DAS) onwards, the plants were tall enough to avoid pooling. Contrary, from this stage samples were collected from three leaf positions, top (T), middle (M), and bottom (B), as schematically visualized in Fig. 1.

The leaf positions were defined as one third of the plant's height. Top leaves represent young leaves, while bottom ones represent old and (partly) senescing leaves. Harvesting took place on two or four (consecutive) days, to split the workload. Therefore, in the text it is referred to the harvest day of Moneymaker, shown in bold in Table 1.

2.2. Protein extraction

Protein extraction from freshly harvested tomato leaves followed the general approach of an alkali protein extraction and pH precipitation as noted in several publications (Tamayo Tenorio et al., 2018; Zhang et al., 2015; Zhang et al., 2014). Therefore, about 10–15 g of tomato leaves per biological replicate were picked, weighted and blended with 100 mL of MilliQ water (Merck Millipore, Darmstadt, Germany) by an Ultra Turrax (T25 & S 25N, IKA Labortechnik/Werke GmbH & CoKG, Staufen, Germany) until complete rupture of the leaf ribs. The suspension pH was adjusted with 2 M NaOH to pH 11 and incubated at 60 °C for 90 min. Subsequently, the suspension was centrifuged (306 g, 5 min), decanted and the proteins present in the supernatant were precipitated by adjusting the pH to 4 with 2 M HCl. A second centrifugation step (3928 g, 10 min) and decanting of the supernatant resulted in a final protein pellet. This pellet was frozen, freeze dried (<−4 °C, <28mTorr, ilShin-BioBase Europe, NL), weighted, and used for further analysis to determine protein yield and pellet purity.

Table 1
Harvesting timepoints for all four different genotypes in days after sowing. In bold the days after sowing to which the following graphs and analysis of this research is referring to.

Genotype/ Developmental stage	Vegetative	Flowering	Fruit forming	Mature fruits (I)	Mature fruits (II)
Moneymaker	**37**	**50**	**77**	**119**	**154**
Gardeners Delight	39	51	79	121	156
S. pimpinellifolium (G1.1596)	37	50	78	120	155
S. pennellii (LA716)	39	51	80	122	157

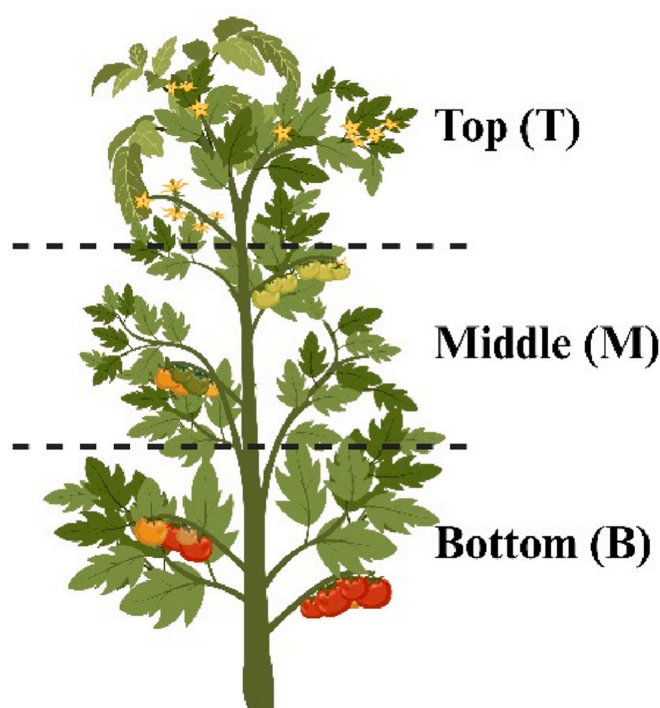

Fig. 1. Scheme of the separation into different leaf positions. From timepoint 77 onwards, tomato plants from all four tomato genotypes were separated by this manner (Created with BioRender.com).

2.3. Quantification of leaf protein, pellet purity and protein yield analysis

Protein content of the initial tomato leaves was determined on a randomly picked subset of the harvested leaf material. These tomato leaves were frozen (−20 °C), freeze dried (<−4 °C, <28mTorr, ilShin-BioBase Europe, NL), and grinded (A11 basic analytical mill, IKA ®, Staufen, Germany) after harvest. The protein purity of the final pellet was also determined after freeze drying. The analysis of protein leaf content and pellet purity was performed with the Dumas nitrogen combustion method using a N exceed analyzer® (Elementar, Langenselbold, Germany). A nitrogen to protein conversion factor of 6.25 was applied (Rhee, 2001) and aspartic acid was used as standard sample. If enough material was present, every sample was measured in triplicate, otherwise in duplicate. For a few samples the protein pellets were very small. If enough material was present, every sample was measured in triplicate, otherwise in duplicate or without technical replicate. In a few cases, Dumas malfunctioning led to missing datapoints, which are indicated by asterisks in figures.

Protein yield was determined as the ratio of protein mass in the final extraction pellet and the protein quantity in the initial leaves from the harvest, as shown in equation (1).

$$Protein\ yield\ [g/g] = \frac{mass\ of\ extracted\ protein\ in\ final\ pellet\ [g]}{mass\ of\ protein\ present\ in\ the\ inital\ leaves\ [g]} \quad (1)$$

2.4. Biochemical analysis of cell walls

Cell wall analysis was performed based on the protocol of Petit et al. (2019), who optimized the benchmark protocol of Pettolino et al. (2012). About 1 g of freeze dried and grinded leaf material was used for the first step of the alcohol-insoluble-residue (AIR) extraction method. The material was washed three times with 36 mL of 80% ethanol and placed for precipitation for 1 h on ice. After that, the material was washed once with 30 mL pure acetone and twice with 30 mL pure methanol to release small molecules, soluble sugars, proteins, lipids, and chlorophyll. An ultracentrifugation step was applied (5 min at 7800 g, Beckmann Avanti, CA, USA) between every washing step. The washing liquid was discarded while the pellet was used for further steps. After drying the pellet in the RapidVap (Labconco, Kansas City, MI, US), starch was removed by an alpha amylase treatment (porcine pancreas, Megazyme, MI, USA) and the pellet was dried again in the RapidVap. This resulted in the intermediate, named AIR, being used for the second step of the cell wall analysis. From the AIR, 20 mg were sampled and hydrolyzed in a two-step sulfuric acid hydrolysis to analyze the monosaccharides composition (rhamnose, arabinose, galactose, glucose, mannose, xylose, galacturonic acid and glucuronic acid). The first step was incubation at 30 °C for 1 h in 72% H_2SO_4 (INNOVA42 incubator, New Brunswick Scientific, Edison, NJ, USA) and the second step was incubation at 103 °C for 1 h in 4% H_2SO_4 (Tuttnauer autoclave 3850 EL-D, Breda, The Netherlands). The monosaccharides, present in the liquid phase, were analyzed via high performance anion exchange chromatography (HPAEC) on a Dionex™ ICS-5000 DC (Thermo Fischer Scientific, Waltham, MS, USA) system. Concentrations of galacturonic acid were on the detection limit, thus not all samples obtained values for galacturonic acid. The concentrations of the carbohydrates were corrected by sugar recovery standards (SRS) to quantify the amount of degraded carbohydrates during the second hydrolysis step. The sulfuric acid hydrolysis and HPAEC analysis was performed on three technical replicates, of which the average was determined and taken as the value of one biological replicate.

The amount of total cell wall per dry matter was defined as shown in equations (2) and (3):

$$Total\ cell\ wall\ /\ dry\ matter\ \ [\%] = \%\ of\ (CW\ /\ AIR) x\ \%\ of\ (AIR\ /\ DM) \quad (2)$$

$$CW\ [mg] = mass\ of\ (Rha + Ara + Gal + Glu + Man + Xyl + GalA + GluA) \quad (3)$$

In the equations above, CW indicates the sum of all measured monosaccharides composing the cell wall polysaccharides (see equation (3)) in mg, AIR the alcohol insoluble residues in mg, DM the dry matter in mg, and Rha, Ara, Gal, Glu, Man, Xyl, GalA, GluA the various cell wall derived monosaccharides (rhamnose, arabinose, galactose, glucose, mannose, xylose, galacturonic acid and glucuronic acid, respectively).

The amount of single cell wall monosaccharides was defined as shown in equation (4).

$$Monosaccharide\ /\ CW\ [\%] = monosaccharide\ [mg]\ /\ CW\ [mg] \quad (4)$$

In the equation above, the monosaccharides are rhamnose, arabinose, galactose, glucose, mannose, xylose, galacturonic acid and glucuronic acid and the CW [mg] is equal as explained previously in equation (3).

2.5. Statistical analysis

Differences in cell wall content and composition across plant age and leaf position were evaluated via one-way analysis of variance (ANOVA) (Significance level $p < 0.05$). As days after sowing (DAS) and leaf positions show collinearity, they were combined into one parameter for the analysis of variance. While DAS explains the plant age, leaf position indicates leaf age. Correlations between the protein extraction yield and the different cell wall carbohydrates were determined through Pearson correlation analysis. Crossed out values are not significantly different from zero. All the analysis were performed in R (Version 4.3.0).

3. Results

3.1. The total amount of cell wall carbohydrates in tomato leaves is stable over plant ages and leaf positions

The total amount of cell wall carbohydrates per dry matter was quantified at different plant ages (DAS), leaf positions (T, M, B) and genotypes (Fig. 2). When comparing the four genotypes, the

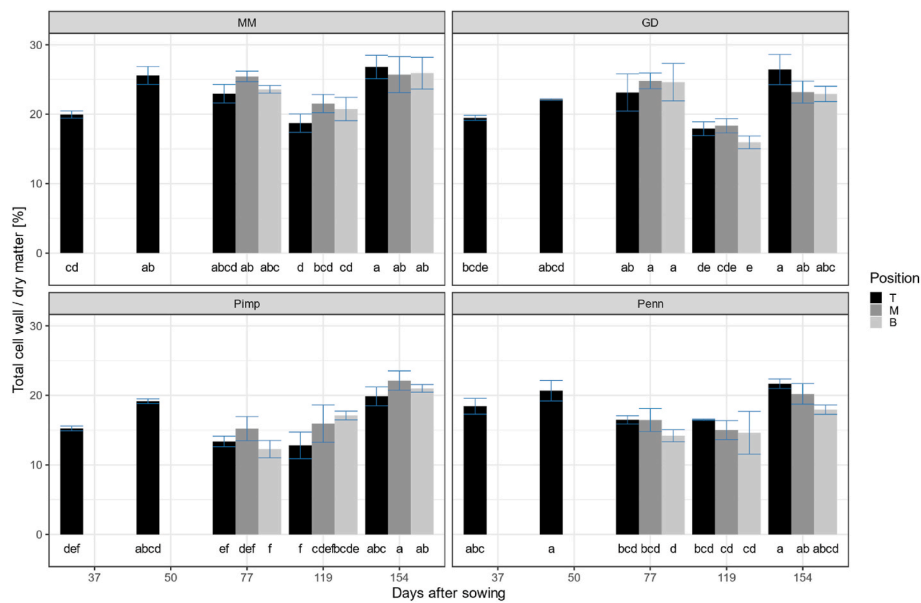

Fig. 2. Relative content of cell wall carbohydrates per dry matter of the four different genotypes Moneymaker (MM), Gardeners Delight (GD), *S. pimpinellifolium* (Pimp) and *S. pennellii* (Penn). Data shown as mean with standard deviation, where the letters indicate statistical significant difference (P ≤ 0.05) between the samples within one genotype.

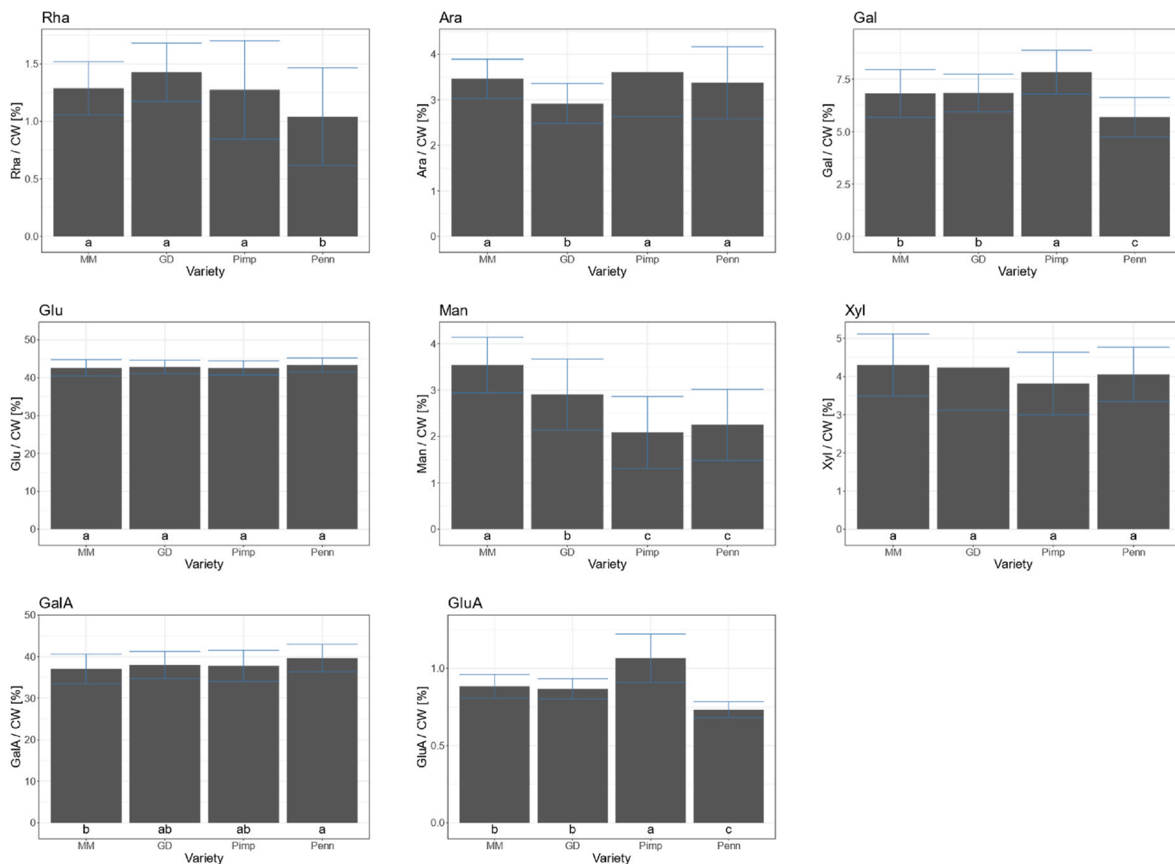

Fig. 3. Percentage of each monosaccharide per total cell wall carbohydrates for the four different genotypes. Each barplot summarizes the percentage of each monosaccharide (Rha = rhamnose, Ara = arabinose, Gal = galactose, Glu = glucose, Man = mannose, Xyl = xylose, GalA = galacturonic acid, GluA = glucuronic acid) over all timepoints (37–154 DAS) and leaf positions (top to bottom) for the four different genotypes (MM = *S. lycopersicum* cv Moneymaker, GD = *S. lycopersicum* cv Gardeners Delight, Pimp = *S. pimpinellifolium*, Penn = *S. pennellii*). Every point represents the average of biological replicates. Letters at the bottom indicate statistical difference between the genotypes (P ≤ 0.05).

S. lycopersicum cultivars Moneymaker and Gardeners Delight contained on average 23.3% and 21.7% total cell wall carbohydrates per dry matter, respectively. These amounts are significantly higher than that observed in the wildtypes, where the total cell wall carbohydrates amounted to 16.7% and 17.5% of dry matter for *S. pimpinellifolium*, and *S. pennellii*, respectively (Fig. 2). Conversely, all the four genotypes showed a similar pattern of dry matter carbohydrates content throughout the different plant ages. Specifically, total cell wall carbohydrates increased in all genotypes from 37 to 50 DAS, followed by a decrease towards the lowest quantities at either 77 DAS (*S. pimpinellifolium* – 13.6%) or 119 DAS (Moneymaker – 20.3%, Gardeners Delight – 17.4%, and *S. pennellii* – 15.3%), to conclude with an increase towards 154 DAS (Fig. 2). At this plant age, the content of total cell wall carbohydrates amounted to 25% for Moneymaker, 25% for Gardeners Delight, 21% for *S. pimpinellifolium*, and 20% for *S. pennellii*. The fold change between the highest and the lowest amount of total cell wall carbohydrates amounted to 1.43, 1.66, 1.81, and 1.53 for Moneymaker, Gardeners Delight, *S. pimpinellifolium,* and *S. pennellii,* respectively.

The *S. lycopersicum* cultivars Moneymaker and Gardeners Delight, as well as *S. pimpinellifolium* showed similar patterns of total cell wall carbohydrates changes over different leaf positions. The cell wall isolated from middle leaves typically contained more carbohydrates than that at the top and at the bottom positions. Conversely, *S. pennellii* represented an exception, with the top position displaying the highest cell wall carbohydrates content over the various plant ages (Fig. 2).

3.2. Glucose and galacturonic acid are the most abundant monosaccharides in cell walls of tomato leaves

The monosaccharide composition of cell wall polysaccharides were quantified in leaves of all tomato genotypes. Generally, glucose (42 ± 2% of cell wall carbohydrates) and galacturonic acid (38 ± 4% of cell wall carbohydrates) were identified as the most abundant monosaccharides across all genotypes (Fig. 3). Conversely, all the other monosaccharides (namely rhamnose, arabinose, galactose, mannose, xylose and glucuronic acid) constituted <10% of total cell wall carbohydrates each in all the genotypes. When focusing on the variability between genotypes, the commercial *S. lycopersicum* cultivars Moneymaker and Gardeners Delight displayed differences only in terms of arabinose and mannose contents. For both of these monosaccharides, Gardeners Delight had a significantly lower content than Moneymaker. Regarding the two wild tomato species, *S. pimpinellifolium* showed a higher content of galactose and a lower content of mannose as percentages of total cell wall carbohydrates, compared to Moneymaker and Gardeners Delight. In addition, the amount of arabinose in *S. pimpinellifolium* did not differ from Moneymaker, but was higher than in Gardeners Delight. *Solanum pennellii,* had the most significant differences compared to all the other genotypes, as it displayed the lowest amount of rhamnose and galactose, and the highest amount of galacturonic acid as percentage of total cell wall carbohydrates.

3.3. Pectin was the cell wall polysaccharide with the largest changes throughout plant ages and leaf positions

In addition to their overall analysis between genotypes, the cell walls carbohydrates were also quantified over different plant ages and leaf positions, per genotype. The most abundant cell wall carbohydrate – glucose – displayed a gradual decrease across both plant ages (from 37 DAS to 154 DAS) and leaf position (from top to bottom) for all the genotypes tested (Fig. 4 – Glu, Fig. S 3 – Glu). For Moneymaker, the decrease was 1.13 fold (45.64–40.27%), for Gardeners Delight 1.10 fold (45.63–41.31%), for *S. pimpinellifolium* 1.10 fold (44.37–40.39%), and for *S. pennellii* 1.08 fold (45.20–41.71%). By contrast, the second most abundant carbohydrate - galacturonic acid – increased over plant ages (from 37 DAS to 154 DAS) and leaf positions (from top to bottom) in all the genotypes. Specifically, for Moneymaker the increase was 1.32 fold (31.18–41.32%), for Gardeners Delight 1.27 fold (32.07–40.85%), for *S. pimpinellifolium* 1.28 fold (30.64–39.07%), and for *S. pennellii* 1.29 fold (32.96–42.39%). Galacturonic acid is a major component of the

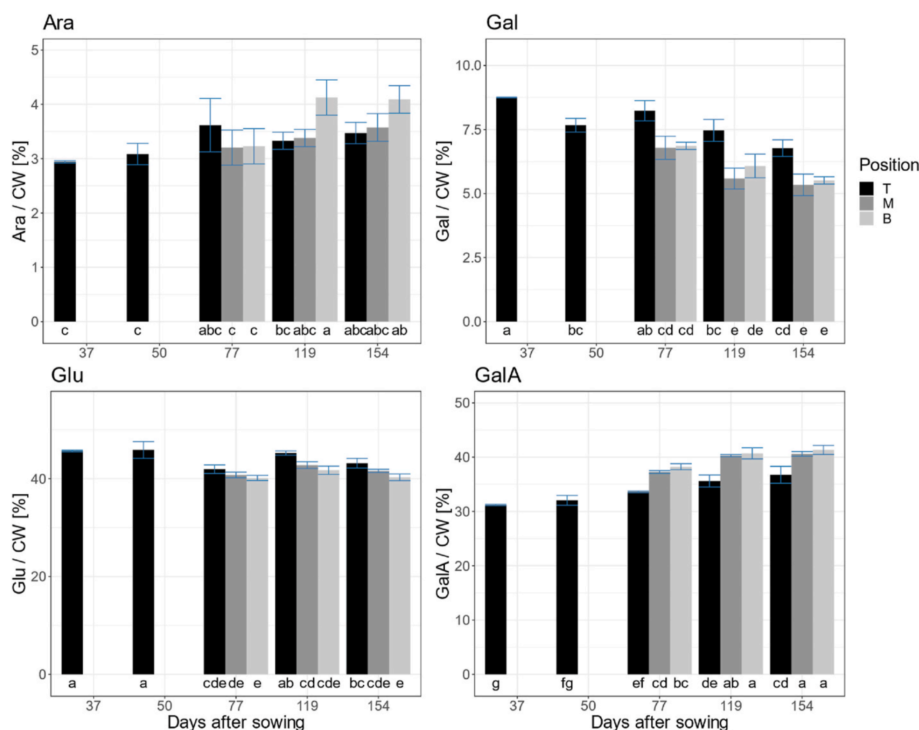

Fig. 4. Monosaccharide concentration of arabinose (Ara), galactose (Gal), glucose (Glu) and galacturonic acid (GalA) as a percentage of total cell wall over different plant ages (DAS) and leaf positions (T = top, M = middle, B = bottom) for Moneymaker. The data of the other genotypes are shown in the supplementary data (Fig. S 3). Letters indicate statistical difference between the samples (combination of DAS and leaf position due to collinearity, P ≤ 0.05).

pectin polysaccharides and other pectin components were thus expected to show similar, increasing trends. While arabinose increased throughout plant ages in all genotypes, galactose showed an opposite behavior, by decreasing from 37 to 154 DAS and from top to bottom leaves in all the genotypes. Specifically, the galactose content in the four genotypes was reduced by 1.59 fold (8.75–5.51%), 1.38 fold (8.24–5.96%), 1.35 fold (9.60–7.11%), and 1.37 fold (7.38–5.40%) in Moneymaker, Gardeners Delight, *S. pimpinellifolium*, and *S. pennellii*, respectively.

3.4. Protein extraction yield from tomato leaves decreases with plant age and leaf position from top to bottom

Next to the biochemical analysis of plant cell walls of tomato leaves, the protein extraction yield across genotypes, plant ages, and leaf positions was also assessed, and the results are shown in Fig. 5. The comparison of the different genotypes, Moneymaker, Gardeners Delight and *S. pimpinellifolium* revealed their similar behavior in protein extraction yields. The highest protein extraction yield was achieved in the youngest material (37 DAS) of these three genotypes, amounting to 57.67 g/g, 55.33 g/g, and 56.76 g/g, respectively (i.e. grams of proteins extracted out of total proteins in leaf material). From this stage on, a decrease in the protein extraction yield was observed, from top to the bottom positions at 154 DAS, where protein extraction yield amounted to 2.19 g/g for Moneymaker, 6.09 g/g for Gardeners Delight, and 1.56 g/g for *S. pimpinellifolium*. The *S. lycopersicum* cultivars Moneymaker and Gardeners Delight did not show significant differences in protein extraction yield between the middle and the bottom positions, suggesting that the lower two plant leaf positions have similar characteristics regarding protein extractability. Contrary, in *S. pimpinellifolium*, differences were shown between the middle and bottom position. Howbeit, for these three genotypes the protein extraction yield is generally more dominantly defined by the leaf position than plant age. In contrast to Moneymaker, Gardners Delight, *S. pimpinellifolium*, and *S. pennellii* showed an overall lower protein extraction yields and somewhat different patterns over leaf positions and ages. Specifically, this genotype displayed the highest yield – 15.3 g/g - at the top position of 77 DAS and the lowest yield – 1.33 g/g - at the middle position of the same plant age (77 DAS). The protein extraction yields from the middle and bottom positions of *S. pennellii* never accounted for more than 4.14 g/g.

While the protein extraction yield is affected by the plant age and leaf position, the protein content present in the leaves stayed more constant (Fig. S 2), in particular for the *S. lycopersicum* cultivars Moneymaker and Gardeners Delight. The wild type genotypes *S. pimpinellifolium* and *S. pennellii* showed significant changes particularly between the leaf positions, however, the correlation between protein content and protein extraction yield remained moderate (Table S 1).

3.5. Pectin composition shows the highest correlations with protein extraction yield

To evaluate if the total content of cell wall carbohydrates and/or the content of specific cell wall monosaccharides over genotypes, plant ages, or leaf positions can have an impact on the protein extraction yield, Pearson correlations between all these variables and protein extraction yield were performed (Fig. 6). The correlations were computed for each genotype separately. The correlations between total cell wall carbohydrates per dry matter ("CW_DM" in Fig. 6) and protein extraction yield ("Yield" in Fig. 6) did not significantly differ from 0 across all the genotypes, indicating that the amount of total cell wall carbohydrates does not display a specific relationship with protein extractability (crossed-out values in Fig. 6).

Conversely, significant and relatively large correlations were found between protein extraction yield and specific cell wall derived monosaccharides. Specifically, the commercial *S. lycopersicum* cultivars Moneymaker and Gardeners Delight both showed strong correlations between different monosaccharides of the pectic carbohydrates and protein yield. These include negative correlations of $r = -0.87$ and $r = -0.86$ between galacturonic acid (GalA) and protein extraction yield for Moneymaker and Gardeners Delight, respectively. These negative correlations reveal that an increase in galacturonic acid, as detected with aging (Fig. 4, Fig. S 3), are accompanied by a decrease in protein extraction yields. Next to galacturonic acid, galactose displayed the second strongest correlations with protein extraction yield – this time positive – for both Moneymaker ($r = 0.78$) and Gardeners Delight ($r =$

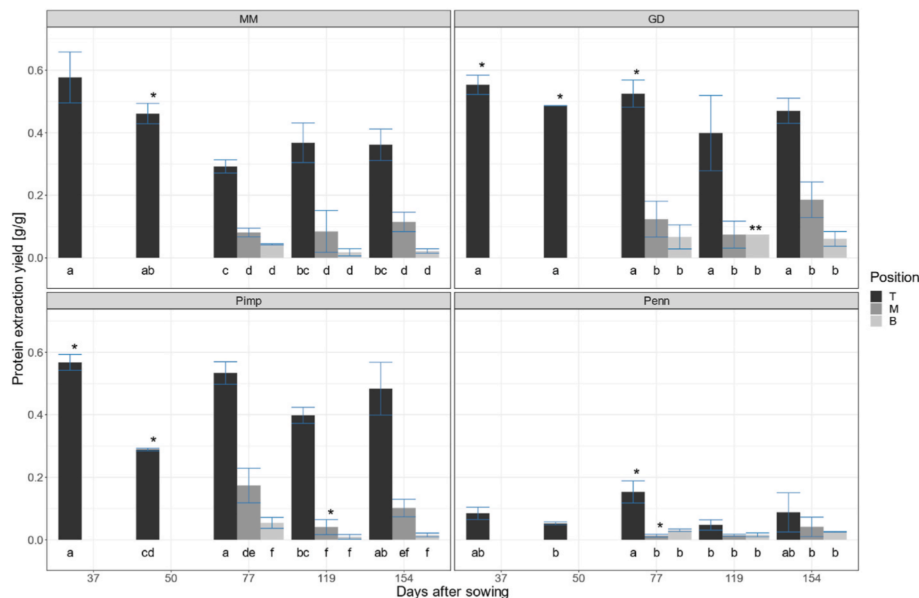

Fig. 5. Protein extraction yield of the four tomato genotypes over the different harvesting timepoints and leaf positions. Each bar indicates the mean of the protein extraction yield, while the error bar indicates the standard deviation for the four different genotypes (*S. lycopersicum* cv Moneymaker = MM, *S. lycopersicum* cv Gardeners Delight = GD, *S. pimpinellifolium* = Pimp, *S. pennellii* = Penn), separated by days after sowing (DAS) and leaf positions (T = top, black; M = middle, dark grey; B = bottom, light grey). Letters at the bottom indicate statistical difference between the samples of one genotype ($P \leq 0.05$).* indicate that only biological duplicates were obtained, ** that no replicate was obtained.

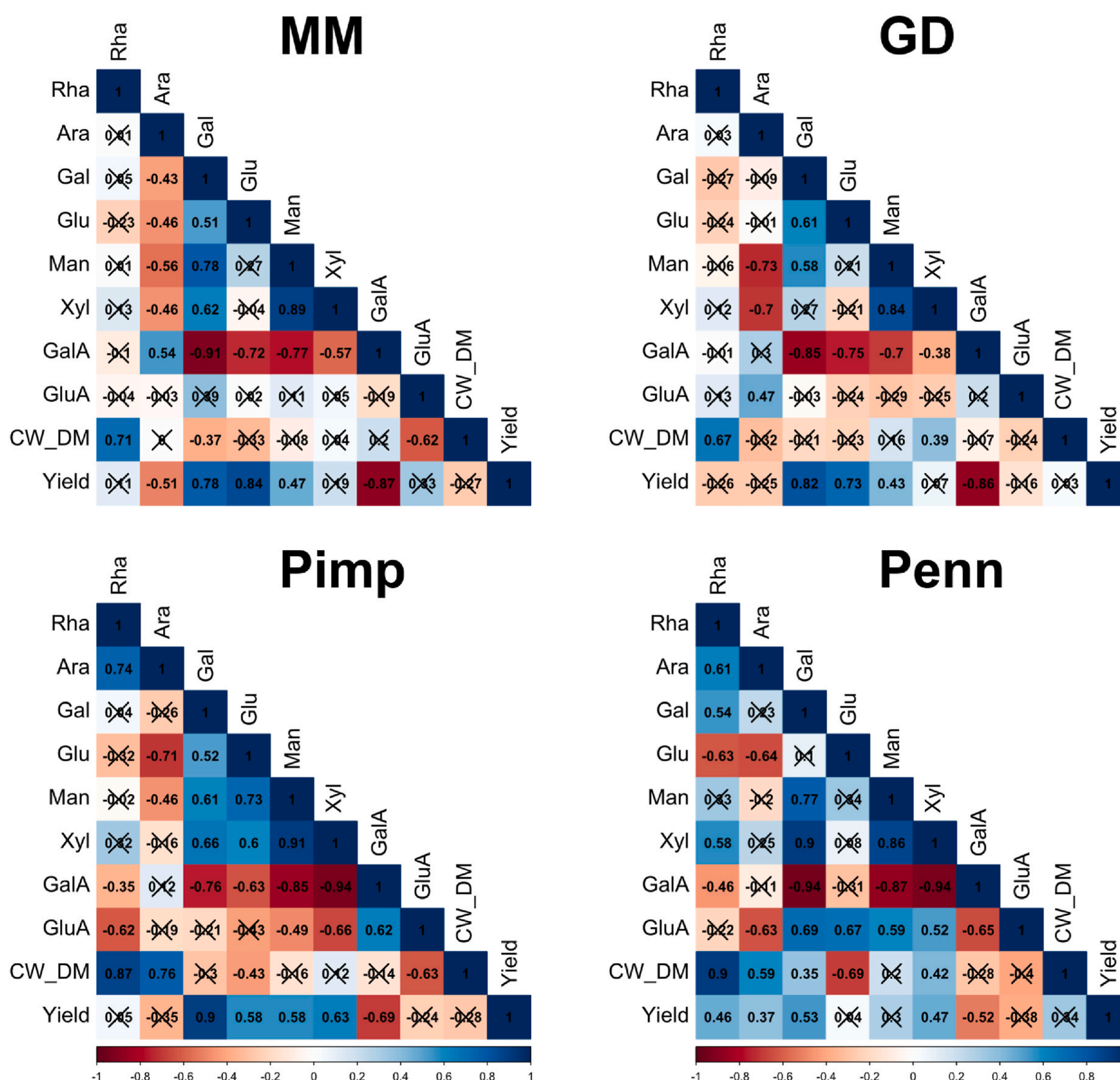

Fig. 6. Correlation plots with Pearson coefficient of protein extraction yield (Yield) against cell wall characteristics. Factors are the different cell wall originated monosaccharides (Rha = rhamnose, Ara = arabinose, Gal = galactose, Glu = glucose, Man = mannose, Xyl = xylose, GalA = galacturonic acid, GluA = Glucoronic acid), and the amount of cell wall per dry matter (CW_DM). Pearson correlation was performed as a pairwise analysis over all timepoints (37–154 DAS) and leaf positions (top to bottom), separated for the genotypes (*S. lycopersicum* cv Moneymaker = MM, *S. lycopersicum* cv Gardeners Delight = GD, *S. pimpinellifolium* = Pimp, *S. pennellii* = Penn*)*. The crossed out values indicate values that are not significantly different from 0.

0.82). Thus, decreases in galactose content observed over plant ageing (Fig. 4) occur also in parallel to decreases of protein extraction yields. Ultimately, the correlations between glucose (the most abundant cell wall carbohydrate) and protein extraction yields were found as the third strongest ones for both Moneymaker (r = 0.84) and Gardeners Delight (r = 0.73), highlighting a similar trend to the one detected for galactose.

Compared to the commercial cultivars, the wildtype genotypes showed (partial) variation in correlation patterns. Specifically, *S. pimpinellifolium* also displayed significant correlations between protein extraction yield and both galacturonic acid (r = −0.69) and galactose (r = 0.89), similarly to Moneymaker and Gardeners Delight. However, the positive correlation observed for galactose outweighed the strength of the negative correlation with galacturonic acid. Moreover, the third-largest correlation in *S. pimpinellifolium* was observed between protein extraction yield and xylose content in cell walls (r = 0.62). Ultimately, *S. pennellii* is the genotype with the lowest observed protein extraction yield (Fig. 5) revealing only moderate correlations between protein extraction yield and cell wall derived monosaccharides. This includes a correlation of r = 0.53 between protein extraction yield and galactose, and a negative correlation of r = −0.52 with galacturonic acid, similarly to what observed in the other genotypes.

4. Discussion

The major aim of this study was to investigate if and how plant cell walls affect protein extraction yield from tomato leaves, since these plant structures are known to represent a physical and chemical barrier to optimal extraction of molecules in several plant species (Safi et al., 2013; Tamayo Tenorio et al., 2018; Rommi et al., 2014; Bals et al., 2009; Sari et al., 2015; Zhang et al., 2015). Therefore, we conducted a comprehensive characterization of the chemical composition of tomato leaf cell walls across different tomato genotypes, plant ages, and leaf positions, and we related the results to the protein extraction yield observed for the same plant materials. The results obtained, pointed out new relationships between cell wall composition and protein extraction yield. Moreover, they revealed differences in the cell wall compositional properties and protein extraction behavior between different tomato genotypes. In this section, the implications of all these findings are

discussed in both a fundamental and applied (breeding) perspective.

4.1. Cell wall content does not affect the protein extraction yield from tomato leaves

A first aspect that was targeted by our research was the investigation of whether the total amount of cell wall carbohydrates in tomato leaf material – irrespectively of their chemical composition – affects the protein extraction yield. In this regard, our results reported significant changes in the total content of cell wall polysaccharides across different plant ages and leaf positions for all the four genotypes analyzed, in a range of ~7% total cell wall carbohydrates per dry matter (Fig. 2). However, no clear pattern over plant age or leaf position was identified for any genotype, an observation supported also by the lack of significant correlations between total cell wall carbohydrates per dry matter (CW_DM) and protein extraction yield (Yield) (Crossed out values in Fig. 6). To our knowledge, this study is the first one to analyze the relationship between total plant cell walls amount and protein extraction yield in tomato, but the lack of a relationship between these two parameters is in line with what observed by Ortega-Regules et al. (2006), who investigated the relationship between tomato cell walls and the extraction of anthocyanins from grape vine. These observations, coupled with the fact that a fluctuation of ~7% total cell wall carbohydrates was observed between the commercial cultivars Moneymaker and Gardeners Delight and the wildtype species *S. pennellii* and *S. pimpinellifolium*, suggests that the total cell wall amount is not a prominent component to consider when aiming at developing crops fitted to the extraction of valuable bioresources from tomato plants, such as proteins. Moreover, in view of these results it seems attainable to combine several breeding goals into single superior tomato genotypes, including better extractability of bioresources and resistance to (a)biotic stresses through thicker cell walls. The latter is a common strategy in (a) biotic stress breeding (Bu et al., 2014; Houston et al., 2016; Cantu et al., 2008), and a preeminent goal in tomato breeding programs (Yang et al., 2018; Asselbergh et al., 2007).

4.2. Modification of cell wall composition is a breeding target for higher protein extraction yields

Next to analyzing the relationship between the dynamics of total cell wall carbohydrates and the protein extraction yield over plant ages, leaf positions, and genotypes, our study also investigated whether changes in the relative composition of cell wall monosaccharides affects the protein extraction yield. In this regard, pectins-derived monosaccharides displayed significant and large correlations with protein extraction yield. Specifically, we observed an increase in arabinose and galacturonic acid levels and a decrease in galactose content during plant aging and at lower plant leaf positions, which were all correlated with variability in protein extraction yield across the genotypes analyzed (Fig. 4). These observations make pectin polymers interesting breeding targets to potentially increase protein extraction yield from tomato leaves. Thus, understanding the metabolic basis of the patterns observed is essential to guide breeding efforts.

Galacturonic acid was found to increase over plant ageing, and such an increase was negatively correlated with protein extraction yield (Fig. 6). This monosaccharide comprises about 70% of the pectin network of plant cell walls (Mohnen, 2008), thus the increase in galacturonic acid is likely due to an increase in pectin content over plant age and positions, which can depend on two main factors. On the one hand, ongoing synthesis of pectins might take place over the whole plant life, leading to their increased accumulation. This was the case in the study by Borniego et al. (2019), where a 1.3-fold increase in total pectins from non-senescing to senescing arabidopsis leaves was detected. On the other hand, the increase of pectins can also be due to the degradation of other cell wall polymers, resulting in an increase of the relative amount of pectins over the total cell wall material. This was shown to be the case in oat leaves, where cellulose and hemicellulose were actively metabolized under induced senescence (Miyamoto et al., 2013). As a conclusion, it can be assumed that both, ongoing pectin generation and degradation of other cell wall polymers occur with increasing plant age and lower plant leaf position, suggesting the limitation of pectin synthesis or cellulose and hemicellulose degradation as a breeding target for higher protein extraction yields from the leafy biomass, given the negative correlation between galacturonic acid content and protein extraction yield (Fig. 6).

Beyond assessing polysaccharide distribution, a dissection of the pectin network into its constituent monosaccharides and an evaluation of their linearity, can give further insights on the effect of cell wall composition on protein extractability. The analysis of distinct pectin monosaccharide ratios offers valuable indications of homogalacturonan content and pectin linearity. Homogalacturonan content, defined as the difference between galacturonic acid and rhamnose (Houben et al., 2011), was found to increase with plant age and lower leaf position in this study (Fig. S 4, supplementary data). This suggests that the rise in pectin content can be attributed to an increase in homogalacturonan. In contrast, pectin linearity, defined as the ratio of galacturonic acid divided by the sum of fucose, rhamnose, arabinose, galactose, and xylose (Houben et al., 2011), increased with plant age but not with lower leaf positions (supplementary data, Fig. S 5). This finding suggests that a more linear pectin network seems to lead to lower protein extraction yields. As homogalacturonan is the exclusive linear polymer within the pectin network, this leads to the assumption that homogalacturonan is the limiting pectin polymer, when aiming for a higher protein extraction yield. This hypothesis is supported by the strong negative Pearson correlations between homogalacturonan content and pectin linearity against protein extraction yields for all genotypes (Fig. S 5, supplementary data). Thus, a lower homogalacturonan content may enhance protein extractability, suggesting further investigation of homogalacturonan synthesis, degradation, and modification.

When focusing on the molecular structure of homogalacturonan, its modifications add another layer of complexity. During the synthesis of homogalacturonan, the C-6 carboxyl group undergoes partial methylation, while the O-2 and O-3 carboxyl groups encounter O-acetylation (Mohnen, 2008). Subsequently, some of the ester residues are removed, facilitating the formation of intermolecular cross-linkages. It is established that de-esterified regions can lead to the formation of cross-linkages, which are regulated by calcium ions and adopt an "egg-box" conformation (Anderson, 2019). These egg-box structures exhibit stiffness and reduce the elasticity of the cell wall (Peaucelle et al., 2011). It is plausible that these rigid structures remain intact during the protein extraction process, resulting in a decrease of protein extraction yield. Hence, we hypothesize that the presence of homogalacturonan and the level of homogalacturonan methylation hinders protein extraction, providing another potential target for breeding efforts towards enhanced protein extraction yields from tomato leaves.

In conclusion, the findings on pectin monosaccharides underscore the significance of these polymers in the context of breeding efforts aiming at improving protein extraction yields from tomato leaves. However, pectin is often part of a highly complex network, whose synthesis involves at least 67 enzymes (Mohnen, 2008), and subsequent modifications, which are strongly influenced by the agile responses to developmental and environmental factors (Gigli-Bisceglia et al., 2020; Vaahtera et al., 2019; Voxeur and Hofte, 2016). Therefore, breeding approaches, which include phenotyping and selection of multiple pectin properties in the leaves, followed by the identification of quantitative trait loci for these traits, can assist when aiming to resolve the puzzle of effects from the pectin network on protein extraction yields.

4.3. Protein extraction yield also depends on factors other than the cell walls

The inclusion of four different genotypes in our study – namely the

two *S. lycopersicum* cultivars Moneymaker and Garderns Delight, *S. pimpinellifolium*, and *S. pennellii* – allowed to inspect relative differences in the relationships between cell wall parameters and protein extraction yields across the genotypes themselves. In this regard, it was noteworthy that while the correlation patterns between protein extraction yield and cell wall properties were similar across the *S. lycopersicum* cultivars Moneymaker and Gardeners Delight, as well as the wild *S. pimpinellifolium* species, *S. pennellii* displayed opposite relationships with respect to several cell wall monosaccharides (Fig. 6). This low correlation can be explained by the low protein extraction yield generated for *S. pennellii*. The low protein extraction yield coupled with a low correlation to cell wall characteristic suggests that other factors than the cell walls play a critical role in protein extraction.

Impacts of physiological factors on protein extraction yield were investigated by Sari et al. (2015). Through the comparison of various biomass sources and examination of their compositions, the authors arrived to the overall conclusion: Protein extraction yield is dominantly determined by 1) the biomass composition, and 2) the biological function of the proteins. In the context of biomass composition, Sari et al. (2015) defined lipids as one of the major bottlenecks on protein extraction. Notably, *S. pennellii*, the genotype exhibiting the lowest protein extraction yields (Fig. 5), has high amounts of epicuticular lipids, accounting for up to 19.9% of the dry weight in older leaves (Fobes et al., 1985). Consequently, it is hypothesized that the abundance of lipids in *S. pennellii* lead to the low protein extraction yields in this genotype. Additionally, the total amount of proteins present in the leaves could affect the protein extraction yield. However, no or only a moderate correlation was identified between these two parameters for Moneymaker/Gardeners Delight and *S. pimpinellifolium/S. pennellii,* respectively (Table S 1). This suggests that the protein content is not a major factor determining protein extraction yield. When considering the biological function of proteins in tomato leaves, the dominant amount of proteins serves functional roles rather than being a storage reservoir. Such functional proteins undergo changes in their overall composition over time. One example is RuBisCo, the most abundant protein particularly in young leaf tissues with a reduction in quantity towards older leaf tissues (Vicente et al., 2011; Yu et al., 2023). A decrease of RuBisCo content is often correlated with a decrease of protein extraction yield, as it is often the primary protein of the final protein concentrate (Santamaría-Fernández and Lübeck, 2020). In opposite to the decrease of RuBisCo content, other proteins, such as senescence associated proteins, increase in their abundance from young to old leaf tissues (Carrion et al., 2013; Mayta et al., 2019; Pruzinska et al., 2017; Roberts et al., 2012). A subset of these senescence associated proteins are proteases. These proteases are involved in the turnover of the proteins present in the leaves, as they degrade leaf proteins in old, senescing leaf tissues, to facilitate nitrogen-reallocation towards younger leaves or fruits (Buet et al., 2019; Lim et al., 2007; Ma et al., 2018). Consequently, a reduction of large proteins occurs, with an increase of small proteins, peptides, and amino acids. Importantly, these small proteins, peptides and amino acids cannot be captured by the protein extraction methods used in this study, as they do not precipitate by pH reduction. Thus, protein extraction from tomato leaves emerges as a complex trait influenced by multiple factors.

To summarize, our data supports the initial hypothesis that cell walls have an effect on protein extraction from tomato leaves. We observed changes in cell wall composition across plant ages and leaf positions and across genotypes. The significant increase in galacturonic acid concentration and the decrease in galactose during aging suggests that the pectin network influences protein extraction. Specifically, the content of homogalacturonan was pointed out to be a key factor. Breeding programs aiming at improving protein extraction yield from tomato leaves should select for varieties with lower amounts of homogalacturonan. Presumably, quantitative trait loci can be identified for the synthesis, modification, and degradation of pectin. Nevertheless, protein extractability from leafy biomass is a complex trait, where further physiological characteristics, such as lipid content, phenol content and protein to peptide ratios, need to be considered when setting up breeding programs.

Disclosure Statement

During the preparation of this work the author(s) used ChatGPT from OpenAI in order to generate a first draft out of bullet points. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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CRediT authorship contribution statement

Marietheres Kleuter: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Yafei Yu:** Writing – review & editing. **Francesco Pancaldi:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Mayra Nagtzaam:** Formal analysis. **Atze Jan van der Goot:** Supervision, Writing – review & editing. **Luisa M. Trindade:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108495.

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