

RESEARCH PAPER

Polymorphism and modulation of cell wall esterase enzyme activities in the chicory root during the growing season

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Abstract

Pectins are major components of the primary plant cell wall. They can be both methylesterified and acetylated and de-esterification occurs by specific esterases. Proteins extracted by NaCl treatment from root cell walls of two chicory varieties (*Cichorium intybus* L. cv. Nausica and Arancha) sampled in an experimental field every 2 weeks between July 2002 and January 2003 were analysed by isoelectrofocalization, semi-denaturing SDS-PAGE, and quantitative assays for their esterase activity. Zymograms showed that chicory root pectin methylesterases belong to a multi-gene family. The isoelectric points of the pectin methylesterase isoforms ranged from pI 3.8 to pI 9.0. Concerning acetyl esterases, only acidic isoforms between pI 4.1 and pI 5.2 were observed, but a large polymorphism of this class of enzymes could be identified in one variety. The results indicate that the root pectin methylesterase activity of the Nausica variety was correlated with ambient temperature, while no significant effect of temperature could be detected on any acetyl esterase isoform.

Key words: Acetyl esterase, cell wall, chicory, cold stress, isoelectrofocalization, pectin, pectin methylesterase, polymorphism, zymogram.

Introduction

Over the last decade, chicory (*Cichorium intybus* L.) has been increasingly used for inulin and fructose production. This crop is sensitive to cold and the degree of polymerization of inulin decreases when a cold stress happens.

Chicory cell walls contain large amounts of pectin. Pectin consists of so-called smooth regions resulting from the linear polymerization of α -1,4-linked galacturonic acid residues interrupted by hairy regions composed of alternating galacturonic acid and rhamnose residues, the latter being more or less substituted at C-4 with galactan and arabinan side chains (Ridley *et al.*, 2001). In a recent model, homopolygalacturonic acid molecules are no more than side chains on a rhamnogalacturonan backbone (Vincken *et al.*, 2003). Whatever model describes pectin most accurately, a variable proportion of the galacturonic acid residues is esterified. Most of the esterification consists of methoxylation at C-6 but some acetylation at C-2 and/or C-3 occurs, especially in the hairy regions. The functional properties of pectins used in the food industry primarily depend on the structural characteristics and, in particular, on the esterification degree of pectin that determines its neutral or acidic character. Preliminary results indicate an average esterification degree of pectin close to 50% in chicory root cell walls and pulp, a by-product of inulin extraction (C Thonar and P Cambier, unpublished results).

Pectin methylesterases (PMEs) control the esterification degree of pectin by removing methylester groups from the C-6 position on the galacturonic acid residues of the pectin backbone. The polymorphism and the abundance of this ubiquitous enzyme at different growth stages in plants suggest that it plays important roles in plant growth and differentiation. Examples of such roles concern fruit maturation and ripening (Steele *et al.*, 1997), microsporogenesis and pollen tube growth (Li *et al.*, 2002; Lacoux *et al.*, 2003; Jiang *et al.*, 2005), seed germination (Ren and Kermodé, 2000), hypocotyl elongation (Bordenave and Goldberg, 1993), tuber yield (Pilling *et al.*, 2002), and root development (Wen *et al.*, 1999). More recently, studies have

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proposed possible roles of PME in systemic movement of tobacco mosaic virus in plants (Chen and Citovsky, 2003).

The three-dimensional structure of plant PME has been determined (Johansson *et al.*, 2002) and consists of a β -helical structure that embodies a central cleft, lined by several aromatic residues, that has been deduced to be suitable for pectin binding. Spatial and temporal regulation of PME activity during plant development is based on a large family of isoforms; in *Arabidopsis*, the PME genes encode pre-pro-proteins and belong to a large multigene family (Richard *et al.*, 1996; Micheli *et al.*, 1998). The pre-region is required for protein targeting to the endoplasmic reticulum and the pro-region is thought to be an inhibitor of the enzymatic activity in preventing protein folding of PME during its secretion to the apoplast (Micheli, 2001). Recently, two proteinaceous inhibitors of PME in *Arabidopsis thaliana* have been described (Wolf *et al.*, 2003; Raiola *et al.*, 2004) and show significant sequence homology to the pro-region of PME. Besides these post-translational regulation mechanisms, PME activity is also regulated by plant hormones (Micheli, 2001). Abiotic stresses such as cold can regulate the transcription of PME. Seki *et al.* (2002) and Kreps *et al.* (2002) have shown, using microarray technology, that the transcription of PME in *Arabidopsis thaliana* was down-regulated by a cold stress.

The mode of action of the enzyme (i.e. random or sequential) is one of the most discussed aspects of PME research and is thought to be under the influence of local cell wall pH, metal ion concentration, and pattern and degree of methylesterification of pectic homopolysaccharide (Goldberg *et al.*, 2001; Willats *et al.*, 2001).

The acetyester groups present on C-2 and/or C-3 of galacturonic residues can be removed by pectin acetyltransferases (AEs). Plant pectin AEs were isolated from orange (Williamson, 1991), mung bean (Bordenave *et al.*, 1995), and from a tomato semi-purified commercial enzyme preparation (Savary, 2001).

As far as is known, neither PME nor pectin AEs has been studied in chicory. While it is known that cold perceived by the plant has direct consequences on the degree of polymerization of inulin, nothing is known about the effect of cold on the structure of pectin. In this study, pectin methylesterases and acetyltransferases extracted from the chicory root cell walls were studied and their activity followed over the growing season.

Materials and methods

Plant material

Chicory was grown in 2002 in an experimental field located at Pottes (B-7660, Belgium) on a sandy loam soil. The two chicory varieties studied (Nausica and Aranacha) have different genetic backgrounds but both varieties are distributed by Chicoline (<http://www.pype-seeds.be/fr/home.html>). Three roots per variety were collected every 2 weeks between July 2002 and January 2003. In the field, roots were washed with MilliQ water and cut in blocks with a sterile knife.

Internal blocks were retained and immediately frozen in liquid nitrogen before storage at -75°C .

Meteorological data

Meteorological data (minimum and maximum air temperatures and rainfall) recorded at a nearby station of the Royal Meteorological Institute of Belgium were obtained for the sampling period (between July 2002 and January 2003). Until the end of September, air temperatures were never much lower than 5°C (Fig. 1). Colder nights ($<5^{\circ}\text{C}$) were observed in October but frost only happened in December and January. Rainfall records are not presented since this parameter did not appear to be statistically correlated to the enzyme activities studied here.

Methods

Wall protein extraction: Blocks originating from a single root at each sampling date were ground in liquid nitrogen and all subsequent steps were carried out at 4°C . Powdered samples (1 g) were resuspended in 5 ml sodium phosphate buffer (2 mM, pH 7.0) and centrifuged (19 000 g for 25 min). The wall-bound proteins were then solubilized from the pellet by incubation for 1 h with a 50 mM succinate:1 M NaCl solution, pH 5. After centrifugation, the supernatant was dialysed overnight against deionized water (Spectra/Por dialysis membrane MWCO: 3500) and concentrated 50 times on a Millipore Amicon-Ultra-5K column. Protein concentrations were determined according to the micromethod of Bradford (1976) using a Bio-Rad kit and BSA as standard. The average wall protein concentration was $300\ \mu\text{g}\ \text{ml}^{-1}$.

Enzymatic activities: PME activity leads to medium acidification in the presence of esterified pectin. This acidification was followed by using the pH indicator Methyl red according to a procedure adapted from Hagerman and Austin (1986). To summarize, esterified pectin from citrus (Sigma P-9561, 1% in 20 mM TRIS-HCl, 5 mM EDTA, 160 mM NaCl at pH 7.6) was incubated with 0.002% Methyl red and root cell wall proteins (final concentration $25\ \mu\text{g}\ \text{ml}^{-1}$). The absorbance was measured every 15 min at 416 nm and 517 nm. Acidification due to PME activity turned the solution colour from yellow to red and the A_{517}/A_{416} ratio increased with time to reach a plateau. The slope of the initial linear part of the curve was used to compare the PME activities of different extracts.

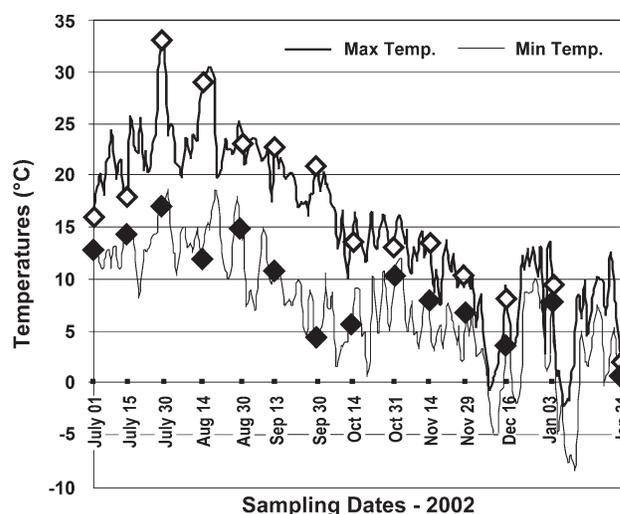


Fig. 1. Minimum (closed symbols) and maximum (open symbols) air temperatures recorded close to the experimental field during the sampling period (between July 2002 and January 2003) at 1 m above the ground.

AE activity in the wall extracts was measured using triacetine (Sigma T-5376) as substrate and its hydrolysis was followed by measuring acetate release. Triacetine (100 mM in 0.025 mM citrate-phosphate buffer, pH 6.0) was incubated with wall proteins (2 µg in 100 µl) at 30 °C for 2 h. After denaturation in boiling water for 5 min, the samples were cooled and the acetate concentrations measured with the Acetic Acid Kit from Roche (ref. S05-0148261) according to the manufacturer's instructions.

Zymograms: Enzyme activities were revealed after electrophoretic separation of the wall protein samples by either isoelectrofocalization (IEF) or semi-denaturing SDS-PAGE. For each zymogram, 4 µg of the proteins extracted by NaCl from root cell walls were loaded per lane.

The IEF were run on a LKB Multiphor II 2117 using Ampholine PAGplate (Amersham Pharmacia) pH 3.5–9.5 for PME and 4.0–6.5 for AE. According to the manufacturer's recommendations, the pre-run conditions for the PME zymograms were 1500 V, 15 mA, 15 W, 20 min, and for the AE zymograms 2000 V, 10 mA, 10 W for 20 min. The running conditions for the PME zymograms were adjusted to 1500 V, 50 mA, 15 W, 90 min, and for the AE zymograms to 2000V, 25 mA, 15 W for 150 min.

Semi-denaturing SDS-PAGE electrophoresis (Michaud and Asselin, 1995) was performed on 8% acrylamide gels for PME and 10% for AE at 4 °C. Precision protein standards (Bio-Rad, 161-0372) were used as molecular weight standards. After migration, SDS was removed with a 2.5% Triton solution for 30 min at room temperature.

In the PME zymograms (Alonso *et al.*, 1995), after IEF and semi-denaturing SDS-PAGE, the gels were equilibrated in a 20 mM TRIS-HCl, 5 mM EDTA, pH 8.5 buffer for 30 min at room temperature. The equilibration buffer was then replaced by a 1% solution of esterified citrus pectin (Sigma, P-9561) in 20 mM TRIS-HCl, 5 mM EDTA, 160 mM NaCl at pH 7.6 and allowed to incubate for 40 min under constant agitation at room temperature. After rinsing the gel in water, the PME activity was revealed by incubation in a 0.05% Ruthenium Red solution followed by overnight discoloration of the gel in water at room temperature.

In the AE zymograms, the AE isoforms were visualized by incubating the IEF or semi-denaturing SDS-PAGE gels at room temperature for 30 min in 94 ml buffer (100 mM phosphate, pH 6.0) containing 100 mg Naphthanyl Diazo Blue B (Sigma, D-9805) and 30 mg α -naphthyl acetate (Sigma, N-8505) dissolved in 6 ml of a 50% (v/v) acetone:water solution.

Statistical analysis

A statistical analysis was performed with the enzymatic activity measured in chicory root samples as the dependent variable and the meteorological data recorded during the growing season as independent variables. Twelve climate factors were considered: the minimum and maximum air temperatures and rainfall (mm) on the day of sampling and their averages over a period of 3, 7, and 14 d before and including the day of sampling. An iterative selection procedure allowed a linear regression model to be built with a large number of variables. From a theoretical point of view, all potential candidate models could be computed in order to compare their respective properties and the joint influence of explanatory variables. However, the number of different candidate models grows steadily with the number of explanatory variables, and the number of observations that had a hand in fitting this model was limited in the present case (only 12 response values for each dependent variable, whereas there were 12 potential explanatory variables). To circumvent this problem, a forward stepwise regression procedure that selected the 'best' subset of variables using an iterative approach was employed here (Neter *et al.*, 1990). Essentially, this search method develops a sequence of regression models, at each step adding or deleting an explanatory variable among the pool of all variables. The criterion for

adding or deleting one variable can be stated equivalently in terms of error sum of squares reduction and coefficient of partial correlation of Fisher's *F* statistic. This search procedure ends with the identification of a single regression model as 'best' model.

Basically, the forward stepwise regression proceeds by selecting a first explanatory variable, based on the fact that the *F* statistic associated with the corresponding simple linear regression model is the largest one. The procedure continues by selecting additional explanatory variables using the same criterion. Additionally, at each step, the procedure tests for the relevance of variables already incorporated into the model at an earlier stage, so that some variables can be dropped subsequently if they are no longer helpful in conjunction with variables added at later stages. The procedure stops when no further variables can either be added or deleted, so that the final model includes the best (most significant) subset of explanatory variables while at the same time being as parsimonious as possible.

Results

Pectin methylesterase

Enzymatic activity: Proteins extracted by NaCl from root cell walls were analysed for their PME activity (Fig. 2). The enzymatic data were first analysed with respect to air temperature since this parameter appeared to be the one with the most correlations. Both Nausica and Arancha had a higher PME activity at the beginning of the season and this activity decreased to a minimum when field temperatures were lowest. The drop in PME activity occurred earlier (13 September) for Nausica whose PME activity was practically always lower than Arancha. Except for sample 8 (31 October), the occurrence of cold consistently maintained the PME activity of Nausica at a low level, while Arancha nearly recovered by the end of the sampling period.

Zymograms: Zymograms obtained after IEF of protein extracts from cell walls of both varieties revealed several bands corresponding to PME isoforms of different

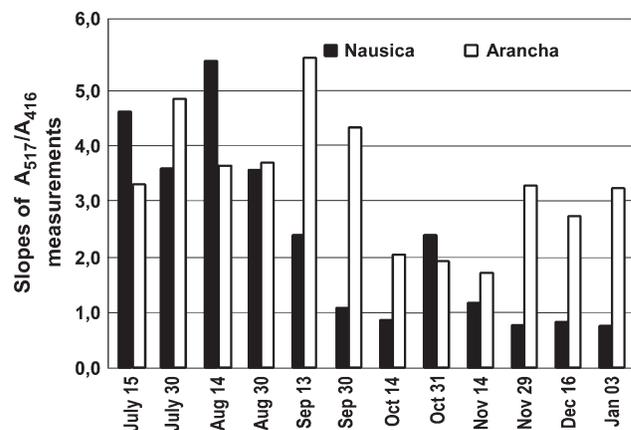


Fig. 2. Colorimetric determination of the PME activity of proteins extracted by NaCl treatment of chicory root cell walls (varieties Nausica and Arancha) sampled at the indicated dates. Identical amounts of wall protein extracts were incubated with methyl esterified pectin in presence of the pH indicator Methyl red. The initial slope of the A_{517}/A_{416} ratio plotted as a function of time is indicated for each sample.

isoelectric points (Fig. 3). Twelve isoforms were observed for the Nausica variety and 11 for Arancha. Acidic and alkaline isoforms could be distinguished in both varieties. Acidic isoforms ($PME_N^{3.8 \rightarrow 5.3}$ and $PME_A^{3.8 \rightarrow 5.7}$) showed a higher activity than more alkaline ones, except for the pI 8.4 isoform. The PME activity was strongest during summer and then decreased consistently, in agreement with colorimetric results. This was especially visible for the Nausica variety, whose PME activity dropped by the end of September with a slight transient recovery in early November. The pI 8.4 isoform showed constant activity throughout the season, while the $PME_N^{5.0}$ isoform was apparently polymorphic, being absent from some samples before the cold stress. The molecular weight of the PMEs was estimated from a zymogram carried out after semi-denaturing SDS-PAGE electrophoresis of a cell wall extract from Nausica chicory root. Figure 4 shows two major bands (PME_2 and PME_3) at about 83 and 91 kDa and a third band (PME_1) with a molecular weight just below 75 kDa.

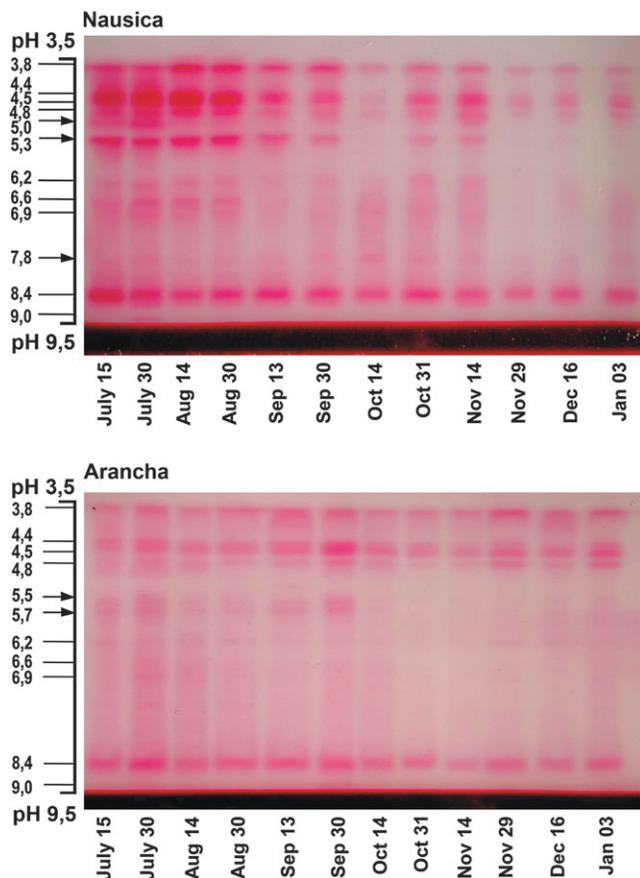


Fig. 3. Zymogram analysis of PMEs after isoelectric focalization of cell wall extracts prepared from Nausica and Arancha chicory root samples. Proteins (4 μ g) from NaCl-treated root cell walls were separated on Ampholine PAGplate between pH 3.5 and 9.5. After equilibration in buffer at pH 8.5 the gels were incubated in a 1% solution of methylesterified pectin at pH 7.6 before coloration of the acidic pectin by Ruthenium red. The isoforms that are specific to one variety are indicated by an arrowhead in the pI scale to the left of the zymograms.

Acetylcysteine

Enzymatic activity: Acetylcysteine activity present in protein extracts of cell walls from both varieties has been quantitatively assayed using triacetone as substrate (Fig. 5). Both varieties showed a relatively constant AE activity throughout the growing season and no clear correlation could be found with field meteorological data. The esterase activity of the Nausica variety was significantly higher as compared with Arancha.

Zymograms: All isoforms of both varieties had acidic pIs between 4.1 and 5.2. The Nausica variety revealed a random variation of the banding pattern from sample to sample (Fig. 6). The Arancha variety was much less heterogeneous regarding its AE activity but the pI 4.3 isoform observed in Nausica was absent from all Arancha samples tested.

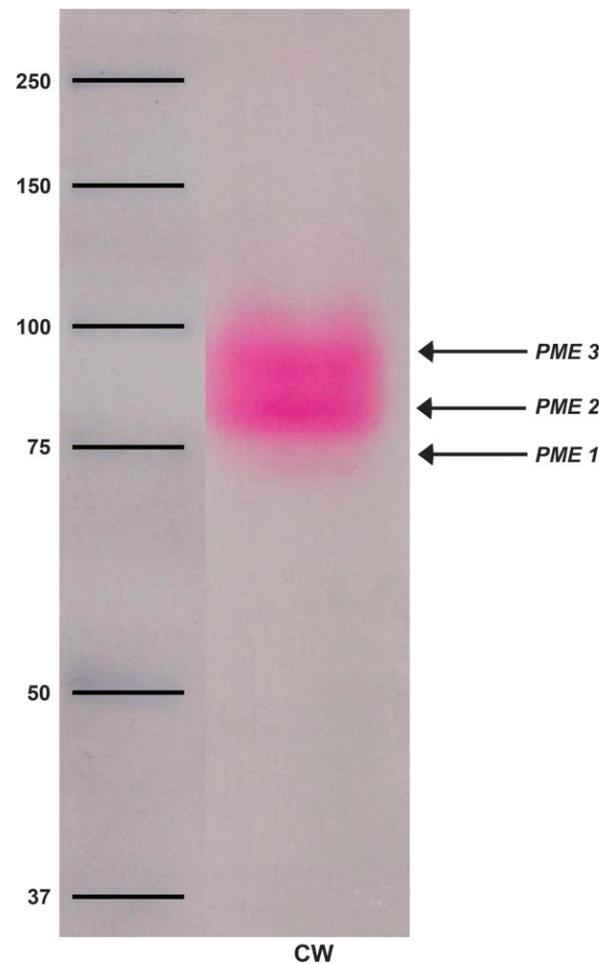


Fig. 4. Zymogram analysis of PME after semi-denaturing SDS-PAGE. Electrophoresis of proteins (4 μ g) from NaCl-treated root cell walls (CW) (variety Nausica) was performed on 8% acrylamide gels at 4 °C. Bio-Rad Precision protein standards were used as molecular weight standards. After migration, SDS was removed by incubating the gel with a 2.5% Triton solution for 30 min at room temperature. Gels were then equilibrated at pH 8.5 before incubation in a 1% solution of methyl-esterified pectin at pH 7.6 and final coloration by Ruthenium red. Arrows point to isoenzymes visible on the zymogram.

No correlation of AE activity could be found with meteorological data for any of the two varieties, which confirms the results obtained with the quantitative assays. When present, the pI 4.2 and pI 5.0 isoforms were almost always the most active ones with $AE_A^{4.2}$ being absent, however, from the last Arancha sample. The molecular weights of the acetyl esterases from Nausica root cell walls were estimated on a zymogram carried out after semi-denaturing SDS-PAGE electrophoresis. Three activity bands labelled *AE1*, *AE2*, and *AE3* were detected at molecular weights of 35, 50,

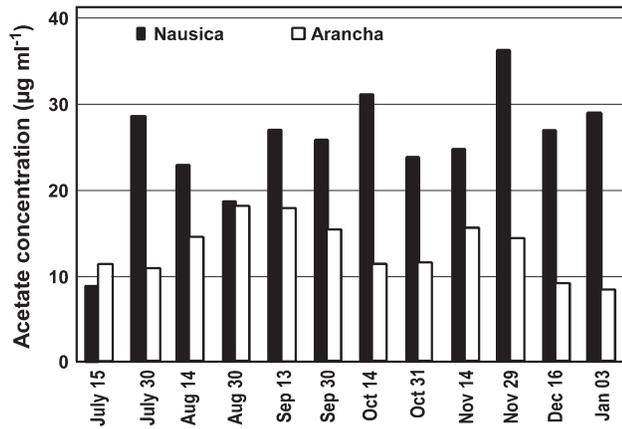


Fig. 5. Acetyl esterase activity of proteins extracted by NaCl treatment of chicory root cell walls (varieties Nausica and Arancha). The activities correspond to acetate concentrations ($\mu\text{g ml}^{-1}$) measured in a 100 mM triacetate solution after a 2 h incubation at 30 °C with the cell wall extracts ($20 \mu\text{g proteins ml}^{-1}$) at pH 6.0.

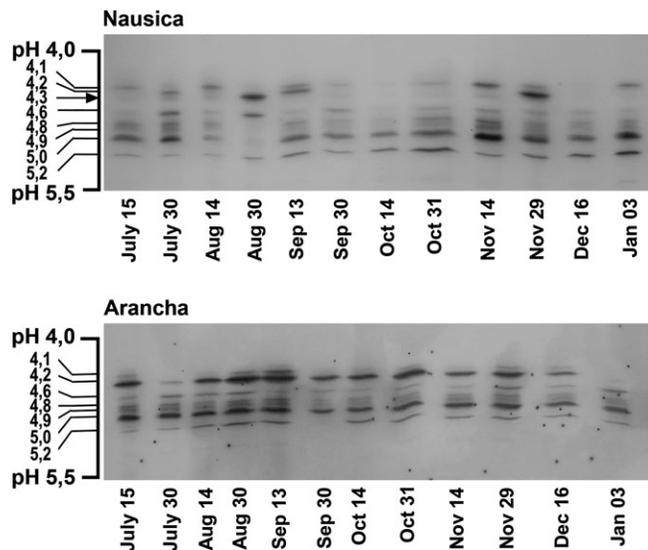


Fig. 6. Zymogram analysis of AEs after isoelectric focusing of cell wall extracts of chicory roots samples from Nausica and Arancha varieties. Proteins ($4 \mu\text{g}$) from NaCl-treated root cell walls were resolved on Ampholine PAGplate pH 3.5–9.5. The electrofocalized gels were incubated at pH 6.0 with Naphthyl Diazo Blue B and α -naphthyl acetate to reveal AE activity. The only isoform specific to Nausica is indicated by an arrowhead in the pI scale to the left of the zymogram.

and 65 kDa, respectively (Fig. 7). The 35 kDa isoform *AE1* was by far the most active on gel.

Statistical analysis

The forward stepwise regression has been applied for both the Nausica and Arancha varieties, where PME and AE activities were the dependent variables to be explained using a subset of the 12 meteorological explanatory variables. The results of the procedure for the selection of the first variable are shown in Table 1, while details for the final selected model are given in Tables 2 and 3. All tests have been conducted using a significance level equal to 15% for Fisher's *F*-test.

From Table 1, it can be seen that for each variable tested separately as first potential variable to be entered into the model (thus corresponding to a simple linear regression model), the results are somewhat different for Nausica and

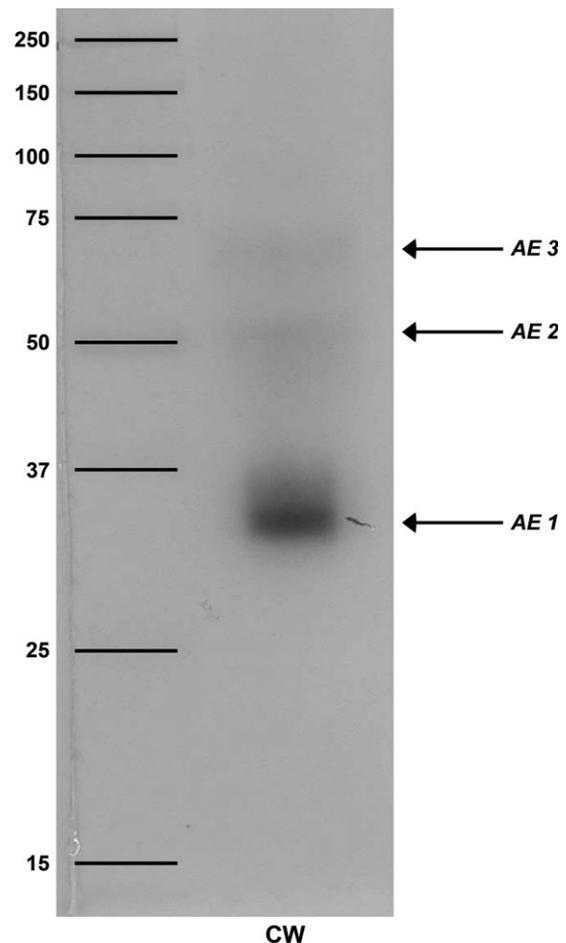


Fig. 7. Zymogram analysis of AEs after semi-denaturing SDS-PAGE. The electrophoresis of proteins ($4 \mu\text{g}$) from NaCl-treated chicory root cell walls (variety Nausica) was performed on 10% acrylamide gels at 4 °C. After migration, SDS was removed by treating the gel with a 2.5% Triton solution for 30 min at room temperature. The gels were then incubated at pH 6.0 with Naphthyl Diazo Blue B and α -naphthyl acetate to reveal AE activities. Arrows point to the isoenzymes visible on the zymogram.

Table 1. Effects of the different meteorological variables (considered separately in a simple linear regression model) on PME and AE activities, both for Nausica and Arancha varieties. Values correspond to the *P*-value associated with the *F*-test.

	PME		AE	
	Nausica	Arancha	Nausica	Arancha
Temperature min (°C)	0.001	0.227	0.084	0.646
Temperature min 3 d (°C)	<0.001	0.167	0.048	0.239
Temperature min 7 d (°C)	0.001	0.067	0.107	0.179
Temperature min 14 d (°C)	0.001	0.099	0.078	0.125
Temperature max (°C)	0.004	0.015	0.314	0.174
Temperature max 3 d (°C)	0.005	0.016	0.331	0.228
Temperature max 7 d (°C)	0.002	0.031	0.211	0.133
Temperature max 14 d (°C)	0.005	0.041	0.192	0.087
Rainfall (mm)	0.118	0.532	0.197	0.073
Rainfall 3 d (mm)	0.066	0.279	0.347	0.063
Rainfall 7 d (mm)	0.098	0.433	0.459	0.289
Rainfall 14 d (mm)	0.84	0.262	0.475	0.792

Table 2a. Results of the forward stepwise regression for PME activity as dependent variable for the Nausica variety

Values correspond to the coefficients and the corresponding *P*-values in the final selected model.

$$\text{PME Nausica} = -0.23 + 0.28 \times \text{Mintemp3}. R^2 = 0.749.$$

	Parameter estimate	Standard error	<i>F</i> -value	Pr > <i>F</i>
Intercept	-0.23	0.52	0.20	0.67
Min temperature of last 3 d (°C)	0.28	0.05	29.78	0.00

Table 2b. Results of the forward stepwise regression for PME activity as dependent variable, for Arancha variety

Values correspond to the coefficients and the corresponding *P*-values in the final selected model.

$$\text{PME Arancha} = 1.53 + 0.10 \times \text{Maxtemp}. R^2 = 0.464.$$

	Parameter estimate	Standard error	<i>F</i> -value	Pr > <i>F</i>
Intercept	1.53	0.66	5.32	0.04
Max temperature on sampling day (°C)	0.10	0.03	8.65	0.01

Arancha varieties. Focusing first on AEs, none of these variables are significant at the 5% level for both varieties. Though no definitive conclusions can be drawn from a separate analysis of each variable effect (as more complex interaction effects could be hidden), this raises some doubts about a clear influence of these meteorological variables on AE activity. For the PME activity, the most influential variables are the various maximum and minimum temperatures. It is also worth noting that rainfall during the last 14 d is by far the least significant variable in all cases.

Pectin methylesterase: For the PME activity, the final subset of variables selected by the stepwise procedure and the corresponding coefficients are shown in Tables 2a and 2b

for both Nausica and Arancha varieties, respectively. It appears that these two varieties exhibit different behaviours with respect to the various meteorological variables.

For the Nausica variety, the only selected variable was the minimum temperature over the last 3 d. The corresponding coefficient is positive, thus showing the positive effect of this variable on the enzymatic activity. The percentage of variance explained by this model as measured by the R^2 statistic is about 75% or, stated in other words, 75% of the total variability of the PME activity is explained by this single variable.

For the Arancha variety, the maximum temperature on the sampling day was the only selected variable, leading again to a simple linear regression final model that only explains about 45% of the total variability. As for Nausica, the coefficient associated with this variable is positive, thus emphasizing the positive effect that temperature has on enzymatic activity. Compared with Nausica, this regression model has a limited explanatory power, as less than half of the variability can be explained by the variables at hand.

Acetylsterase: For the AE activity, the final subset of variables selected by the stepwise procedure and the corresponding coefficients are shown in Tables 3a and 3b, with an explanatory power of the models which is quite different.

For Nausica, two variables were finally selected: minimum and maximum temperature over the last 3 d. For maximum temperature, the associated coefficient is positive, whereas it is negative (but not very significantly) for minimum temperature. Only 50% of the total variability is explained by this model, so that it can be considered as having a weak explanatory power.

For Arancha, the two selected variables are rainfall during the last 3 and 7 d. However, only 50% of the total variability is explained by this model, thus showing again that, as for PME, meteorological variables have a limited impact on enzymatic activity for this variety. According to the results given in Table 1 and the corresponding discussion, it is also clear that the incorporation of rainfall into the final model is only due to the high selected value of 15% for the *F*-test; selecting a 5% level would have led to the conclusion that none of these variables has a significant impact on AE activity.

With respect to the limited explanatory power of these models and the limited amount of data at hand, no clear cut conclusions can be drawn for AE activities from this statistical analysis. The only satisfactory model is obtained for PME activity of Nausica that emphasizes the positive effect of temperature on this enzyme activity. Globally, Nausica and Arancha varieties exhibit quite different sensibilities with respect to minimum temperatures, the contrast being much more marked for PME than for AE. While Nausica PME activity is largely influenced by this variable, Arancha is rather insensitive. As for AE activity, Arancha can even be considered to be insensitive to it.

Table 3a. Results of the forward stepwise regression for AE activity as dependent variable, for the Nausica variety

Values correspond to the coefficients and the corresponding *P*-values in the final selected model.

$$AE \text{ Nausica} = 27.3 - 1.84 \times \text{Mintemp}3 + 0.82 \times \text{Maxtemp}3. R^2 = 0.516.$$

	Parameter estimate	Standard error	<i>F</i> -value	Pr > <i>F</i>
Intercept	27.3	4.13	43.67	<0.0001
Min temperature last 3 d (°C)	-1.84	0.66	7.84	0.02
Max temperature max last 3 d (°C)	0.82	0.45	3.36	0.10

Table 3b. Results of the forward stepwise regression for AE activity as dependent variable, both for the Arancha variety

Values correspond to the coefficients and the corresponding *P*-values in the final selected model.

$$AE \text{ Arancha} = 13.9 - 2.03 \times \text{Rainfall}3 + 1.44 \times \text{Rainfall}7. R^2 = 0.508.$$

	Parameter estimate	Standard error	<i>F</i> -value	Pr > <i>F</i>
Intercept	13.9	1.02	184.1	<0.0001
Rainfall last 3 d (mm)	-2.03	0.76	7.24	0.02
Rainfall last 7 d (mm)	1.44	0.74	3.73	0.09

Discussion

The structure and biochemical composition of pectin directly determine its physico-chemical properties and pectic enzymes present in the cell wall remodel and eventually degrade pectin in a developmentally controlled manner. Both transient remodelling (e.g. during cell growth) and wall disassembly (e.g. during xylem formation, fruit maturation, or abscission) are concerned. Pectin solubilization and depolymerization are caused by a range of pectinases among which PME's play a pivotal role, preparing the molecule for further degradation by polygalacturonases and pectate lyases. In this work the cell wall esterase activity was measured in chicory roots of two different varieties cultivated for inulin production. The esterase activities were followed all through the growing season and analysed with regard to meteorological data.

The PME activity found in root cell wall extracts was much higher at the beginning of the growing season. Air temperatures below 5 °C resulted in a drop of PME activity observed by both colorimetric assays and zymograms, with this activity drop being stronger in the Nausica variety. A statistical analysis indicated that 75% of the variation of PME activity in Nausica could be explained by the average minimum air temperature of the last 3 d before sampling. Results obtained from Arancha did not show such a strong correlation with any of the climatic factors considered. Cold was thus the factor most correlated with repression of PME activity in Nausica. In other words, Nausica was more responsive to cold than Arancha.

Since roots are buried in the ground and the temperature variations are damped by the mass of soil, the perception of cold spells could take place in the leaves before a signal is transmitted to the root. Signal transmission from leaves has been amply documented in the case of daylength perception as a floral stimulus transmitted to the shoot apex (Mouradov *et al.*, 2002) and in the case of systemic acquired resistance. As far as is known, cold modulation of PME activity is a totally new example of cold control on plant cell physiology.

During cold, one expects secretion to the cell wall to be reduced, and the activity of most PME isoforms (12 for Nausica and 11 for Arancha) had indeed decreased by the end of the season, but one basic isoform at pI 8.4 (PME_N^{8.4} and PME_A^{8.4}) remained active in both cultivars over the whole year, even after freezing temperatures were recorded. The permanent activity of this isoform can result from a lower turnover in the cell wall and/or from a continuous secretion of this isoform specifically by Golgi vesicles fusing to the plasmalemma, whatever the external air temperature. Cold treatment adversely affects protein transport through the secretory pathway, protein transport being slowed down or even arrested at low temperatures (Bar-Peled and Raikhel, 1997). However, PME activity in Nausica roots clearly dropped as early as mid-September when the aerial parts of the plants had not yet experienced any night colder than 7 °C and when maximum day temperatures regularly exceeded 20 °C. It could thus be concluded that transcription, and/or translation but probably not secretion, of PME isoforms in roots were controlled by air temperature signalled to the root.

Responses to cold stress are complex, involving numerous changes in gene expression, metabolism, and morphology. Changes in membrane fluidity and calcium influxes appear to be the earliest events leading to cold stress signal transduction (Browse and Xin, 2003) and various transcription factors are involved in the regulation of these stress-inducible genes (Shinozaki *et al.*, 2003). Later changes include the increased expression of many genes, the reduction or the cessation of growth, transient increases in abscisic acid concentrations, changes in membrane lipid composition, accumulation of compatible osmolytes, and increased levels of antioxidants. The precise level at which cold controls PME activity in chicory roots (i.e. transcriptional, translational, or post-translational) has not been addressed in this study.

Proteins of basic pI's are positively charged at the physiological pH of 5.6 usually reported for plant cell walls and they can therefore interact with negatively charged pectins. Few acidic PME isoforms are reported in plants (Komae *et al.*, 1990; Bordenave *et al.*, 1995; Li *et al.*, 2002). Which respective roles do these alkaline and acidic isoforms play in plant cell walls? Neutral and alkaline isoforms of PME from mung bean hypocotyls are active on methylesterified pectins and show a marked

decrease of activity at pH 5.6 when the degree of esterification of the pectic substrate falls below 50–60% (Goldberg *et al.*, 2001). It also appears that chains with long blocks of unesterified polygalacturonic acid can act as competitive inhibitors of PME, depending on the pH of the incubation medium. Functional PMEs can thus coexist in cell walls with methylesterified pectins. Acidic isoforms of pI lower than 5.6 are negatively charged in the cell walls and therefore do not interact as tightly with acidic pectin as basic isoforms, although they still need a concentrated salt treatment for extraction. These acidic PMEs could maybe further de-esterify pectins that are no longer processed by neutral and alkaline isoforms, explaining the existence of both acidic and alkaline PME isoforms in the cell wall.

Why does cold stress act in a differential way on acidic isoforms as compared with basic ones? In the presence of basic isoforms only, pectins would not be completely de-esterified and they would consequently be less hydrated than if additional PME isoforms were present and able to turn remaining methylesterified uronic residues into hydrated anions. A lower hydrated wall is certainly an advantage for a biennial plant whose root has to over-winter even in frozen soil.

Concerning AEs, eight acidic isoforms were identified on zymograms. Nausica and Arancha are so-called open pollination varieties and thus neither hybrids nor clones. The diversity of enzyme patterns observed between samples could simply be due to genetic polymorphism within the population and not to any environmental factor. The Nausica cultivar was indeed derived from 12 elite plants, whereas the Arancha cultivar was based on two components only, which could explain the higher polymorphism of the Nausica AE zymograms.

The nature of the AEs present in the cell walls is still not clear; the zymogram used is not specific for acetylated pectins and only detects acetylerase activity. Several esters other than methylesters and acetylestes exist in plant cell walls like the borate-diol-esters that allow dimerization of two molecules of rhamnogalacturonan-II and the feruloylestes found on galactose and arabinose residues of pectin side chains (Levigne *et al.*, 2004). Further work is needed to determine the exact substrate specificity of the AE isoforms observed in the chicory cell walls.

In conclusion, the chicory root cell wall contains numerous isoforms of both PME and AE and temperature seems to control PME activity in one cultivar but not AE activity. The significance of this polymorphism and its implication in the properties of the root cell walls still need to be explained.

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