The chemical identity of intervessel pit membranes in *Acer* challenges hydrogel control of xylem hydraulic conductivity

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Abstract. Ion-mediated enhancement of the hydraulic conductivity of xylem tissue (i.e. the ionic effect) has been reported for various angiosperm species. One explanation of the ionic effect is that it is caused by the swelling and shrinking of intervessel pit membranes due to the presence of pectins and/or other cell-wall matrix polymers such as heteroxylans or arabinogalactan–proteins (AGPs) that may contain acidic sugars. Here, we examined the ionic effect for six *Acer* species and their pit membrane chemistry using immunocytochemistry, including antibodies against glycoproteins. Moreover, anatomical features related to the bordered pit morphology and vessel dimensions were investigated using light and electron microscopy. The ionic effect varied from 18 % (± 9) to 32 % (± 13). Epitopes of homogalacturonan (LM18) and xylan (LM11) were not detected in intervessel pit membranes. Negative results were also obtained for glycoproteins (extensin: LM1, JIM20; AGP glycan: LM2), although AGP (JIM13)-related epitopes were detected in parenchyma cells. The mean vessel length was significantly correlated with the magnitude of the ionic effect, unlike other pit or vessel-related characteristics. Our results suggest that intervessel pit membranes of *Acer* are unlikely to contain pectic or other acidic polysaccharides. Therefore, alternative explanations should be tested to clarify the ionic effect.

Keywords: *Acer*; glycoproteins; hydraulic conductivity; immunocytochemistry; ionic effect; pectic polysaccharides; pit membrane; vessel.

Introduction

According to the cohesion–tension theory, long-distance water transport in plants occurs through the xylem tissue in a passive way (Askenasy 1895; Dixon and Joly 1895; Jansen & Schenk 2015). The driving force for water uptake is set by the transpiration rate in leaves and is under stomatal control (Damour et al. 2010). In angiosperm xylem, individual vessel elements dissolve their primary and secondary cell wall partially to form perforated, multicellular vessels that are specialised for water transport. However, these stacks of vessel elements are of finite length, which means that no individual vessel provides a direct connection from the roots to the canopy of a tree. Instead, water is transported through an interconnected network of vessels, which is enabled by...
thousands of bordered pits between neighbouring vessel walls (Choat et al. 2008).

The micromorphology of bordered pits between adjacent vessel walls and especially the intervessel pit membrane is assumed to play a key role in drought-induced embolism formation (Lens et al. 2011; Scholz et al. 2013a; Schenk et al. 2015) and regulating hydraulic resistance (Sperry et al. 2005; Wheeler et al. 2005; Choat et al. 2006).

Several studies reported that xylem hydraulic conductance may depend on the pH, the ionic strength, and ionic identity of the perfused solvents (Zimmermann 1978; van Ieperen 2007; Nardini et al. 2012). Our mechanistic understanding of this so-called ‘ionic effect’, however, remains limited. Frequently cited explanations for the ionic effect include the hydrogel hypothesis (Zwieniecki et al. 2001) and the electroviscosity hypothesis (van Doorn et al. 2011; Santiago et al. 2013), which both rely on chemical and physical properties of intervessel pit membranes.

According to the hydrogel hypothesis, the resistance of the water molecules through the porous network of pit membrane microfibrils is affected by a potential swelling or shrinking of pectins, which are a highly heterogeneous class of acidic polysaccharides (Bonner 1946; Caffall and Mohnen 2009; Kastner et al. 2012). The backbone of pectins is a linear chain of (1-4)-linked D-galacturonic residues (homogalacturonan, HG), which can be modified in various ways, most notably by methylesterification to generate acidic residues. Unesterified galacturonic residues of pectic HG can interact with cations in the xylem sap and have already been applied on bordered pit membranes (Table 1), immunocytochemistry techniques are limited to few species only, especially Populus and Vitis vinifera. Most studies show that pectic polysaccharides are absent in the actual, fully developed pit membrane, but present in the outermost rim of the pit membrane (i.e. the annulus) and in immature pit membranes of developing vessel elements (Table 1; Wydra and Beri 2007; Plavcová and Hacke 2011; Kim and Daniel 2013; Herbette et al. 2015). An exception to this is the report of pectins based on JIM7 in juvenile shoots of Vitis vinifera (Sun et al. 2011). Moreover, the ionic effect did not decrease in transgenic plants of Nicotiana tabacum (PG7 and PG16) with reduced HG content in comparison with wild-type plants with assumingly higher pectin levels (Nardini et al. 2007a). If pectin is lacking in intervessel pit membranes, it is possible that other acidic cell-wall matrix polysaccharides/proteoglycans, such as heteroxylans or AGPs, could show a similar swelling and shrinking behaviour as pectins (Li and Pan 2010), although there is also little evidence for their distribution in intervessel pit membranes (Table 1; van Doorn et al. 2011). Xyloglucan (LM15) and mannan (LM21) epitopes were found to be absent in mature bordered pit membranes of hybrid poplar and hybrid aspen (Kim and Daniel 2013; Herbette et al. 2015), while xyloglucan was found in intervessel pit membranes of juvenile grapevine stems and European aspen (Sun et al. 2011; Kim and Daniel 2013).

This paper aims to further test the hydrogel hypothesis by investigating the ionic effect and the chemical composition of pit membranes in six closely related Acer species. Because most of the earlier evidence indicates that HG-related pectic epitopes (JIM5, JIM7, LM7, LM 19 and LM20) and rhamnogalacturonan (RG)-I-related pectic epitopes (LM5 and LM6) could not be detected in intervessel pit membranes (Table 1), we limited our selection of HG-related antibodies to LM18, which has not been applied to pit membranes as far as we know, while LM11 was chosen as a heteroxylan antibody, and four antibodies (LM1, LM2, JIM13 and JIM20) were selected to test for the presence of glycoproteins, including extensin and AGP glycans. Glycoproteins have not been reported in the actual membrane of bordered pits (Wydra and Beri 2007; Herbette et al. 2015), although proteins and AGPs occur in xylem sap and may accumulate in pit membranes of vessel elements and tracheids (Iwai et al. 2003; Buhtz et al. 2004). Whether or not (glyco)proteins play a role in the ionic effect is unknown (Neumann et al. 2010).

Previous studies suggest that pectins disappear during the final stages of vessel development by hydrolytic enzymes that remove the non-cellulosic components (O’Brien 1970; Kim and Daniel 2012; Kim and Daniel 2013; Herbette et al. 2015). Although about 20 different antibodies have already been applied on bordered pit membranes (Table 1), immunocytochemistry techniques
Table 1. Overview of antibodies tested on pits in angiosperm xylem tissue. PM = intervessel pit membrane; Par PM = parenchyma pit membrane; HG = homogalacturonan; RG = rhamnogalacturonan-1; Me = methylesterified; AGP = arabinogalactan-protein; + = strong to weak signals detected; -- = negative staining.

| Antibody | Epitope | Reference | Species | PM annulus | Parenchyma PM | Comment |
|----------|---------|-----------|---------|------------|---------------|---------|
| 2F4 | Non-Me galacturonic acid blocks dimerized by calcium | Herbette et al. 2015 | Populus tremula x alba | * | + | + | * = Only immature pits |
| JIM5 | Partially Me-HG/de-esterified HG | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | | + For parenchyma and vessel walls after incubating with Ralstonia solanacearum strain To-udk2 |
| JIM7 | Partially Me-HG | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | | + For parenchyma and vessel walls after incubating with Ralstonia solanacearum strain To-udk2 |
| LM5 | (1→4)-β-galactan side chains of RG | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | Parenchyma cell walls |
| LM6 | (1→5)-α-l-arabinan of RG | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | |
| | | Herbette et al. 2015 | Populus tremula x alba | | | |
| | | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | |
| | | Plavcová and Hacke 2011 | Betula papyrifera, Populus balsamifera, Prunus virginiana, Amelanchier alnifolia | | | |
| | | Sun et al. 2011 | Vitis vinifera var. Chardonnay and var. Riesling | | | |
| | | Herbette et al. 2015 | Populus tremula x alba | | | |
| | | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | | | |
| | | Plavcová and Hacke 2011 | Betula papyrifera, Populus balsamifera, Prunus virginiana, Amelanchier alnifolia | | | |

Continued
### Table 1. Continued

| Antibody | Epitope | Reference | Species | PM annulus | Parenchyma | Comment |
|----------|---------|-----------|---------|------------|------------|---------|
| LM7      | Non-blockwise de-esterification of HG | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | – | – | |
| LM19     | Low Me HG epitopes | Kim and Daniel 2013 | Populus tremula × P. tremuloides, P. tremula | +* | +* | + | * = Only immature pits |
| LM20     | High Me HG epitopes | Kim and Daniel 2013 | Populus tremula × P. tremuloides, P. tremula | +* | +* | + | * = Only immature pits |
| RU1      | RG      | Herbette et al. 2015 | Populus tremula × alba | +* | + | + | * = Only immature pits |

2. Non-cellulosic, non-pectic polysaccharides

| Antibody | Epitope | Reference | Species | PM annulus | Parenchyma | Comment |
|----------|---------|-----------|---------|------------|------------|---------|
| BMG C6   | Galactoglucomannan | Kim and Daniel 2012 | Populus tremula | | | |
| LM10     | Xylan   | Kim and Daniel 2012 | Populus tremula | All cell walls in the xylem |
| LM11     | Xylan   | Kim and Daniel 2012 | Populus tremula | All cell walls in the xylem |
| LM15     | Xyloglucans | Plavcová and Hacke 2011 | Betula papyrifera, Populus balsamifera, Prunus virginiana, Amelanchier alnifolia | + | + | + |
|          |         | Kim and Daniel 2013 | Populus tremula × P. tremuloides, P. tremula | +* | +* | + | * = Only immature pits |
|          |         | Herbette et al. 2015 | Populus tremula × alba | – | – | |
| CCRC-M1  | Fucosylated xyloglucan | Sun et al. 2011 | V. vinifera | + | + | – |
| LM21     | Mannan  | Kim and Daniel 2012 | Populus tremula | | | |
|          |         | Herbette et al. 2015 | Populus tremula × alba | – | – | |

3. Plant cell-wall proteoglycans/glycoproteins

| Antibody | Epitope | Reference | Species | PM annulus | Parenchyma | Comment |
|----------|---------|-----------|---------|------------|------------|---------|
| AX1      | arabinoylanx | Herbette et al. 2015 | Populus tremula × alba | | | Only pit borders |
| LM2      | AGP     | Wydra and Beri 2007 | Solanum lycopersicum (genotype L390 and L7996) | Metaxylem vessel walls |

4. Lignin

| Antibody | Epitope | Reference | Species | PM annulus | Parenchyma | Comment |
|----------|---------|-----------|---------|------------|------------|---------|
| Anti-S   |         | Herbette et al. 2015 | Populus tremula × alba | + | + | + Also for pit borders |
Therefore, we expect that two scenarios could explain the magnitude of the ionic effect in six Acer species: (1) the chemical identity and/or the anatomy of intervessel pit membranes, or (2) none of these two, which means that alternative explanations would be required.

**Methods**

**Plant material**

We collected 1- to 3-year-old branches from five Acer species (*Acer campestre*, *A. monspessulanum*, *A. palmatum*, *A. sieboldianum* and *A. tataricum*) from single trees at the botanic garden of Ulm University during April and August 2012. Between April and August 2012, we also collected branches from eight trees of *A. pseudoplatanus* at the same location. Sample collection for all species took place between 8 and 9 am to avoid severe water stress levels and high levels of native embolism. Although minor changes in the effect of seasonality cannot be completely excluded, differences in the ionic effect between April and September were found to be insignificant (Gascó et al. 2007). Moreover, pit membrane chemistry has been reported to differ between the growing and the non-growing season (Wheeler 1981; Pesacreta et al. 2005), but is unknown to show considerable differences between spring and summer. All trees sampled were older than ten years. Collected branches were cut in the field and transported to the lab in a plastic bag with wet tissue within ten minutes. For all experiments, branches were recut under water prior to measurements. For the anatomical measurements and immunolocalisation, we focused on the last (i.e. current year) growth ring.

**Vessel length measurements**

We used the silicone injection method to assess the vessel length distribution (Sperry et al. 2005; Scholz et al. 2013b). Five branches per species were collected and trimmed to a length of 30 cm. The branches had a minimum diameter of 8 mm. Stem segments were perfused at 0.175 MPa with commercial bottled water (Auvergne Regional Park, France) at room temperature for 30 min, or until no air bubbles could be seen at the open end. We used a two-component silicone system (Rhodosil ESA 7250 A and ESA 7250 B, Bodo Müller GmbH). Both substances were mixed in an 11:1 ratio (A to B). The colourless silicone was stained by adding 1 % (w/v) Uvitex (Ciba UK plc, Bradford, UK) dissolved in chloroform. The silicone mixture was degassed for 20 min, or until no gas bubbles emerged. Silicon was injected in the stem segment with a Modell 100 pressure chamber instrument (PMS, Oregon,
USA). Small amounts of the silicon mixture were poured in glass vials. The distal end of branches were submerged in the silicon mixture and transferred to a pressure chamber, which was then pressurized to 0.2 MPa for 2 h. The silicon was allowed to polymerize for 2 h at room temperature and transverse sections were made with a sliding microtome (GLS, Birmensdorf, Switzerland). Vessel length distribution was assessed by investigating these sections, starting at the proximal end. The first positive silicone observation in a vessel was considered to represent the maximum vessel length. We used the maximum vessel length to calculate four additional distances to estimate the vessel length distribution, with 6 mm as the minimal distance (Sperry et al. 2005).

Hydraulic measurements of branch segments

Commercial bottled water (Auvergne Regional Park, France) was used as a reference solution for our hydraulic measurements to avoid artefacts caused by low salt concentration (Sperry et al. 2005; van Ieperen 2007). According to data from the supplier, this reference solution included 0.504 mM Na\(^+\), 0.286 mM Ca\(^2+\), 0.07 mM Mg\(^2+\), 0.158 mM K\(^+\), 0.084 mM SO\(_4^{2-}\) and 1.58 mM HCO\(_3^-\), while the pH was 7. Samples were perfused with this solution at 0.2 MPa for at least 30 min, or until no air bubbles emerged from the open end. This flushing was required to refill embolised conduits. As the magnitude of the ionic effect is influenced by the percentage of intact conduits, we tested the ionic effect by comparing stem-specific conductivity (Sperry et al. 2006), the stem-specific hydraulic conductivity (K\(_s\), kg s\(^{-1}\) m\(^{-1}\) MPa\(^{-1}\)) was measured on stem segments that corresponded to 80% of the average vessel length (ranging from 2.27 to 4.81 cm), which means that most vessels were closed and had no open vessel ends in the sample. This approach allowed us to make a direct comparison across the six Acer species, because this method takes into account the distribution of the vessel length classes (Gascó et al. 2006). We perfused the samples with a pressure of 0.007 MPa in a Sperry apparatus (Sperry et al. 1988). The flowrate of water was monitored each 5 s with an Sartorius CPA 225D balance. If the flow rate showed less than 5% variation over 30 s, the flow was considered to be stable and the hydraulic conductance was measured over 1 min. In most cases, stable flow rates were obtained after 15 min. We tested the ionic effect by comparing stem-specific conductivity (K\(_s\)) with the reference solution and a high-salt solution, which consisted of commercial water with an additional 25 mM KCl, and calculated the ionic effect (%) as the increase in conductivity. The xylem surface area was measured after the hydraulic measurements were completed. Callipers were used to measure the xylem diameter, which allowed us to calculate the xylem surface area. The pith area could be neglected because of its small area in our stem segments.

Immunolocalisation of cell-wall components

Fluorescence microscopy was applied using a set of six rat monoclonal antibodies: LM18 (pectic HG; Verherbruggen et al. 2009), LM11 (heteroxylan; McCartney et al. 2005), LM2 (AGP glycan; Smallwood et al. 1996; Yates et al. 1996), JIM13 (AGP glycan; Knox et al. 1991), JIM20 (extensin; Smallwood et al. 1994; Knox et al. 1995) and LM1 (extensin, Smallwood et al. 1995). As far as we know, this is the first study applying the antibodies LM1, LM18, JIM20 and JIM13 to intervessel pit membranes. Possible masking effects restricting access to cell-wall components (e.g. Marcus et al. 2008) were not considered in this study.

Slivers of wood from the current growth ring were fixed overnight in a solution with 4% paraformaldehyde, 0.1 mM phosphate buffer and 1% sucrose at pH 7.3. Samples were embedded in LR-Gold resin following the instructions of the manufacturer. We tested cell-wall epitope distribution by fluorescence microscopy. Semi-thin sections (0.5 \(\mu m\)) were mounted on an object slide and incubated for 30 min in 5% (w/v) milk protein in phosphate-buffered saline (PBS). A 5-fold dilution of the primary antibody in milk/PBS replaced the blocking milk. After an hour, we washed the sample three times with PBS. The secondary antibody goat anti-rat-IgG conjugate with fluorescein isothiocyanate (FITC) was diluted 100-fold and incubated in darkness for 1 h in milk/PBS. Unbound antibody was removed by washing samples three times with PBS for 5 min. The sections were heat-fixed to glass slides without a mounting medium. We included controls to test non-specific binding of the secondary antibody. Additionally, we tested the sample for auto-fluorescence, which was quenched by staining with toluidine blue. Samples showing no auto-fluorescence were treated with calcofluor white to enhance the contrast of cell walls.

Wood anatomy

Wood anatomical features related to the dimensions and quantity of pits and vessels were measured following standard protocols (Scholz et al. 2013b). Transverse sections with a thickness of ca. 25 \(\mu m\) were prepared with a sliding microtome (GLS, Birmensdorf, Switzerland). Staining of the sections was performed with a 1% (w/v) safranin solution in 50% ethanol and a 1% (w/v) alcian blue solution in demineralised water. After staining, the samples were dehydrated in an ethanol series (50%, 70% and 96%), treated with Neo-clear clearing agent (Merck Millipore, Germany), and embedded in Neo-mount (Merck Millipore, Germany). The embedding
medium was polymerized in an oven at 60 °C overnight. Photographs of the latest growth rings were taken with a Leica DM RBE microscope system (Leica Microsystems, Wetzlar, Germany).

Electron microscopy, including scanning electron microscopy (SEM) and transmission electron microscopy (TEM), was applied to investigate ultra-structural details of pits and cell walls. Tangential sections of about 1 cm² and a maximum thickness of 3 mm were oven dried overnight and mounted on SEM stubs using carbon cement. The stubs were sputtered with a thin layer of gold using a Balzers Union sputter coater (Lichtenstein, Lichtenstein). SEM pictures were obtained with a Zeiss DSM 942 SEM-system (Jena, Germany).

For TEM observations of the pit membrane thickness (T PM) and vessel wall thickness (T VW), slivers from short branch segments (5 mm) were transferred to Karnovsky’s fixative at room temperature. After washing with 0.1 M phosphate buffer, samples were postfixed in 1 % buffered osmium tetroxide (OsO4) for 4 h at 5 °C. The OsO4 was removed by washing with phosphate buffer and a graded ethanol series (30 %, 50 %, 60 %, 70 %, 90 % and 96 % ethanol) was applied to dehydrate the samples. The ethanol was then gradually replaced with Epon™ resin over several days. The samples were cut with an ultramicrotome (Ultracut, Reichert-Jung, Austria) to obtain transverse, semi-thin sections of 500 nm. Ultrathin sections were observed with a Jeol JEM-1400 TEM (München, Germany).

**Statistical analysis**

Anatomical characters and hydraulic measurements (Ks and ionic effect) were expressed by average values (± standard deviation) based on at least five measurements per species. The correlation between a xylem feature anatomical and the ionic effect was tested by calculating a Pearson correlation coefficient with P = 0.05 as a threshold value. The IBM SPSS Statistics version 20 (2011, SPSS Inc., Chicago, IL, USA) was used for the analyses.

**Results**

**Hydraulic measurements of branch segments**

The stem specific hydraulic conductivity (Ks) using the reference solution varied from 0.269 (± 0.018) kg s⁻¹ m⁻¹ MPa⁻¹ (mean ± SD) in A. monspessulanum to 0.367 (± 0.024) kg s⁻¹ m⁻¹ MPa⁻¹ in A. sieboldianum. All species showed a significant increase in Ks when perfusing the samples with the 25 mM KCl solution (see [Supporting Information — Table S1] and Fig. 1). The ionic effect measured was on average 24.7 % (± 12.4) and ranged from 18.0 % (± 9.7) in A. palmatum to 32.4 % (± 13.1) in A. tataricum (Fig. 1).

**Immunolocalisation of cell wall polysaccharides**

Results from the immunolocalisation are summarized in Table 2. The controls included for non-specific binding of the secondary antibody were negative for all species studied. No positive staining could be detected for LM1 (extensin), LM2 (AGP glycan), JIM20 (extensin) and LM18 (HG; Fig. 2). From the six antibodies tested, LM11 (xylan, Fig. 3) and JIM13 (AGP, Fig. 4) showed positive staining of the xylem tissue. The antibody LM11 labelled each xylem cell wall, indicating the ubiquitous but weak distribution of xylan in their secondary cell walls.

The JIM13 (AGP glycan) epitope was present in ray and axial parenchyma cells, but most pronounced in vessel-associated parenchyma cells of all six Acer
species. Its distribution was consistently observed near the inner cell wall of vessel-associated parenchyma cells (Fig. 4). The staining intensity varied between the species studied (Table 3). In A. palmatum, the JIM13 epitope was clearly seen in parenchyma cells (Fig. 4A–C), while weaker signals were detected in A. sieboldianum (Fig. 4D–F) and A. tataricum (Fig. 4G–I).

Anatomical observations

A survey of the anatomical characters of the xylem cells is provided in Table 3. The mean vessel diameter (D) was consistent for most species and around 25 μm, except for A. pseudoplatanus, which showed a more variable and wider diameter of 42 (±11) μm. The latter species also showed the lowest vessel density (Vd) with 82 (±13) vessels per mm², and the thickest intervessel walls (Tv), which were 6.4 (±1.1) μm. Acer campestre, A. monspessulanum and A. palmatum showed vessel density values (Vd) around 120 vessels per mm², while A. sieboldianum and A. tataricum had more than 180 vessels per mm².

Differences in the solitary vessel index (Vd) ranged from 0.82 for A. campestre, which means that 82% of all vessels counted were solitary, to 0.43 in A. pseudoplatanus. The vessel-grouping index (Vg) ranged between 1.27 in A. tataricum and 1.84 in A. sieboldianum. The average vessel length (Lv) varied from 2.27 (±0.13) cm in A. palmatum to 4.81 (±0.18) cm in A. tataricum, while Lv was around 3 cm for A. monspessulanum, A. pseudoplatanus and A. sieboldianum.

Little variation was found for the intervessel pit-field fraction (FPF), with values ranging from 0.65 (±0.01) to 0.71 (±0.02) in A. monspessulanum and A. tataricum, respectively. The average surface area of a single intervessel pit membrane (APm) was between 21.23 (±3.4) μm² in A. tataricum and 34.18 (±6.6) μm² in A. pseudoplatanus. The pit membrane thickness (TPM) varied considerably, with relatively thin membranes of 146 (±16) nm in A. tataricum to 235 (±25) nm in A. pseudoplatanus. The pit aperture area (APa) showed a constant intraspecific variation (SD = ±0.3 μm), except for A. pseudoplatanus, which also had the largest pit aperture area (APa) of 2.95 (±0.93) μm².

We found a strong correlation between the average ionic effect and the mean vessel length (Lr) (Pearson’s r = 0.84, n = 6, P = 0.03). No other anatomical features (D, Dh, Vd, Vg, AP, APm, FPF, TPM and FP) correlated with the ionic effect values of the six Acer species.

Discussion

Testing the hydrogel hypothesis

One of the key findings of this paper is that none of the epitopes for the six antibodies tested could be detected in intervessel pit membranes of the six Acer species studied. The lack of HG and RG-I-related epitopes in intervessel pit membranes as based on LM18 and seven additional antibodies tested in previous studies (Table 1; Plavcová and Hacke 2011; Kim and Daniel 2012, 2013; Herbette et al. 2015; but see Sun et al. 2011) suggest that pectic polysaccharides appear to be absent in intervessel pit membranes of fully developed vessels, and that the hydrogel hypothesis does not fully explain the ionic effect. Therefore, an alternative hypothesis is required, which supports our scenario 2 as outlined in the Introduction, but rejects scenario 1 (Nardini et al. 2007b, 2011; van Doorn et al. 2011; Santiago et al. 2013). New functional explanations for the ionic effect could for instance come from surfactants and surfactant-coated nanobubbles in xylem sap, which may change in size depending on the ionic concentration of xylem sap (Duval et al. 2012; Jansen and Schenk 2015; Schenk et al. 2015). A lack of pectins and removal of the non-cellulosic, non-pectic components during vessel development was also suggested based on traditional staining techniques (O’Brien and Thimann 1967; O’Brien 1970). However, the presence of pectins in vessel-parenchyma pit membranes has been reported several times (Table 1; Plavcová and Hacke 2011; Kim and Daniel 2012; Kim and Daniel 2013; Herbette et al. 2015), indicating that pit membrane chemistry also depends on the pit type. While the occurrence of pectins in vessel-parenchyma pit membranes could be associated with gel and tylosis formation (Rioux et al. 1998; De Micco et al. 2016), these pectins are unknown to have any effects on the ionic effect. We could not detect pectins in the half-bordered vessel-parenchyma pits of Acer (via LM18).
The presence of cellulose in fully developed intervessel pit membranes was proven in functional assays and based on histological observation with specific probes for crystalline and non-crystalline cellulose (Dusotot-Coucaud et al. 2014; Herbette et al. 2015). Few studies, however, have suggested that methyl-esterified pectins remain present in mature intervessel pit membranes of Acer pseudoplatanus, Dianthus caryophyllus, Populus italica and Robinia pseudo-acacia, while acidic pectins and vic-glycol side-groups are removed from pit membranes during hydrolysis (Catesson et al. 1979; Catesson 1983). The presence of acidic pectins has been reported in torus-bearing pit membranes of Ulmus (Czaninski 1979; Jansen et al. 2004). Based on the ruthenium red staining and hydroxylamine-ferric chloride staining techniques, the relative abundance of acidic versus methylesterified pectins was suggested to be closely related to the ionic effect in four Lauraceae species (Gortan et al. 2011). Light microscopic observation after staining with ruthenium red also suggested that pectins occur in the intervessel pit membranes of Umbellularia californica (Nardini et al. 2011). However, these more

Figure 2. Selection of lack of epitope detection for the antibodies LM2 (AGP glycan, A and B), LM1 (extensin, C and D), JIM20 (extensin; E and F) and LM18 (HG; G and H) applied to transverse wood sections. Species include A. tataricum (A–C, G and H) and A. sieboldianum (C and D, E and F). Micrographs on the left (A, C, E and G) show the sections stained with calcofluor white, while fluorescence images are shown on the right (B, D, F and H). V, vessel; *Ray parenchyma cells. Scale bar = 100 μm.
Figure 3. Selection of immunohistological observations with the anti-xylan antibody (LM11) in A. monspessulanum (A) and A. palmatum (C). Micrographs on the left (A and C) show the transverse wood sections stained with calcofluor white, the localisation of the antibody under fluorescent light on the right (B and D). No positive staining can be seen in the vessels (V), ray parenchyma (*), and intervessel walls (arrows). Scale bar = 100 μm.

Figure 4. Selection of immunohistological observations with the anti-AGP antibody (JIM13) in A. palmatum (A and B), A. sieboldianum (C and D) and A. tataricum (E and F). Micrographs on the left (A, C and E) show the transverse wood sections stained with calcofluor white, the fluorescence images are shown on the right (B, D and F). Positive signals (arrows) for AGP can be seen in axial parenchyma cells (*) associated with vessels (V), ray parenchyma, but not in intervessel pit membranes (triangles) Scale bar = 100 μm.
Table 3. Wood anatomical features of six Acer species. All numbers represent mean values ± SD. * = no standard deviation is given for $V_0$ and $V_5$, which were measured on 100 individual vessels in two to three transverse wood sections. A. Cam, a. Campestre; a. Mon, a. Monspeussulanum; a. Pla, a. Platanatum; a. Pse, a. Pseudoplanus; a. Sie, a. Sieboldianum; a. Tar, a. Tataricum. Character acronyms follow [Supplementary Table 2].

| Character (units) | A. cam | A. mon | A. pla | A. pse | A. sie | A. tat |
|------------------|--------|--------|--------|--------|--------|--------|
| $A_p$ (mm$^2$)   | 0.60 ± 0.24 | 0.36 ± 0.12 | 0.37 ± 0.12 | 0.61 ± 0.24 | 0.63 ± 0.18 | 0.73 ± 0.2 |
| $A_{pp}$         | 2.06 ± 0.45 | 1.75 ± 0.40 | 1.66 ± 0.35 | 1.68 ± 0.28 | 2.03 ± 0.47 | 1.55 ± 0.42 |
| $A_{pip}$ (µm$^2$) | 23.29 ± 5.04 | 25.23 ± 3.61 | 18.54 ± 2.70 | 34.18 ± 6.62 | 19.15 ± 3.24 | 21.23 ± 3.37 |
| $A_{pip, ap}$ (µm$^2$) | 1.99 ± 0.37 | 1.34 ± 0.35 | 1.24 ± 0.32 | 2.95 ± 0.93 | 1.62 ± 0.34 | 0.97 ± 0.27 |
| $A_T$ (mm$^2$)   | 3.10 ± 0.83 | 2.01 ± 0.53 | 1.97 ± 0.52 | 2.84 ± 0.81 | 2.84 ± 0.72 | 3.40 ± 0.77 |
| $D$ (µm)        | 23.39 ± 5.68 | 21.07 ± 5.39 | 27.65 ± 6.45 | 41.19 ± 11.16 | 29.86 ± 7.44 | 22.48 ± 5.03 |
| $D_0$ (µm)      | 28.28 ± 6.26 | 26.23 ± 6.81 | 33.14 ± 7.73 | 42.32 ± 11.16 | 34.56 ± 8.61 | 27.14 ± 6.08 |
| $F_C$            | 0.28 ± 0.05 | 0.28 ± 0.05 | 0.29 ± 0.04 | 0.31 ± 0.08 | 0.32 ± 0.04 | 0.30 ± 0.05 |
| $F_{LC}$         | 0.18 ± 0.03 | 0.32 ± 0.03 | 0.37 ± 0.06 | 0.57 ± 0.08 | 0.42 ± 0.02 | 0.20 ± 0.01 |
| $F_P$            | 0.19 ± 0.04 | 0.18 ± 0.03 | 0.19 ± 0.03 | 0.21 ± 0.03 | 0.22 ± 0.03 | 0.22 ± 0.03 |
| $F_{PF}$         | 0.69 ± 0.05 | 0.65 ± 0.01 | 0.66 ± 0.05 | 0.69 ± 0.06 | 0.69 ± 0.04 | 0.71 ± 0.02 |
| $L_C$ (cm)       | 0.60 ± 0.07 | 0.94 ± 0.06 | 0.77 ± 0.09 | 1.63 ± 0.17 | 1.28 ± 0.06 | 0.96 ± 0.04 |
| $L_v$ (cm)       | 4.21 ± 0.46 | 3.04 ± 0.20 | 2.27 ± 0.13 | 2.86 ± 0.30 | 3.12 ± 0.05 | 4.81 ± 0.18 |
| $T_{Pw}$ (nm)    | 188 ± 37 | 225 ± 29 | 201 ± 40 | 235 ± 26 | 192 ± 11 | 146 ± 16 |
| $T_{VW}$ (µm)    | 2.63 ± 0.59 | 3.52 ± 0.82 | 2.48 ± 0.48 | 6.427 ± 1.07 | 2.17 ± 0.51 | 1.97 ± 0.49 |
| $V_0$ (mm$^2$)   | 125 ± 27 | 120 ± 40 | 104 ± 28 | 83 ± 13 | 187 ± 11 | 239 ± 27 |
| $V_6$            | 1.33 | 1.54 | 1.64 | 1.52 | 1.84 | 1.27 |
| $V_5$            | 0.82 | 0.68 | 0.63 | 0.43 | 0.58 | 0.80 |

traditional staining techniques should be interpreted with caution because the classical reaction of ruthenium red with pectins is typical but not highly specific (Bonner 1946; Luft 1971).

Evidence for the lack of pectic polysaccharides in intervessel pit membranes based on immunocytochemical techniques appears to be consistent (Table 1). Application of a commercial pectinase treatment to intervessel pit membranes in Fagus sylvatica did not affect the pit membrane ultrastructure in TEM, and was found to have no effect on embryo resistance, unlike cellulase-treated material (Dusotoit-Coucaud et al. 2014). The observation that hydrolysis of pectins induced a sharp increase in vulnerability to embolism, without any significant effect on hydraulic conductance, could be caused by the occurrence of pectins in the pit membrane annulus (Plavcová and Hacke 2011; Kim and Daniel 2013). However, methyl-esterified HG and fucosylated xyloglucons (XYGs) were detected in intervessel pit membranes of grapevine plants based on the JIM7 and CRCC-M1 antibodies, respectively (Sun et al. 2011). A potential explanation for this finding of pectins in intervessel pit membranes could be that the observations by Sun et al. (2011) are based on juvenile xylem of young branches (< 12 weeks old), which may include a high amount (ca. 35 %) of living, not fully differentiated vessels (Jacobsen et al. 2015). The observation of methylsterified HG and XYGs in grapevine should, therefore, be tested in mature xylem tissue.

Heteroylans (LM11) appear to be abundantly distributed in the secondary cell wall of all xylem cells (Awano et al. 2002). The presence of the JIM13 AGP epitope characterises xylem parenchyma cells, including both ray and axial parenchyma (Fig. 2), while LM2 targets a different AGP epitope that appears to be absent. AGPs have also been reported in meta- and protoxylem vessels of Echinacea purpurea using antibodies against (1→3-D-Glc)3 Yariv phenylglycoside (Göllner et al. 2013). AGPs perform various functions in plants: they are involved in growth, programmed cell death, pattern formation, and interact with growth regulators (Seifert and Roberts 2007). In some parenchyma cells, we detected AGPs in the plasma membrane based on the JIM13 epitope. The appearance of AGPs in the plasma membrane is a logical consequence of glycosylphosphatidylinositol (GPI) anchoring in the
plasma membrane. Although more evidence is required, AGPs could be involved in the monitoring of the hydraulic system, formation of tyloses, refilling of embolised conduits or other hydraulic processes.

To what extent do anatomical features account for the ionic effect?

Surprisingly, we found a positive and significant correlation between the mean vessel length and the ionic effect, but not with any other vessel and bordered pit characteristics. This finding suggests that species with longer vessels such as A. tataricum have a stronger ionic effect than species with a shorter mean vessel length. For Populus tremula, Tilia cordata and Acer platanoides, a negative correlation between xylem conduit diameter and ionic effect has been reported (Aasamaa and Söber 2010). Considering that vessel diameter and length are positively related (Hacke et al. 2006), these findings appear not to agree with our measurements. Hence, further research based on a larger number of species and a wide range of vessel lengths would be required to test the correlation reported here.

The lack of other anatomical correlations appears to contradict earlier work on four Acer species (Nardini et al. 2012), including three species that were also investigated in this study (i.e. A. campestr, A. monspessulanum and A. pseudoplatanus). A positive correlation was found between the ionic effect and characters related to vessel grouping and intervessel connectivity (Jansen et al. 2011; Nardini et al. 2012). A correlation between the ionic effect of four Acer species and the intervessel contact fraction (F_c) was only supported at the interspecific level and not significant at the intraspecific level (Nardini et al. 2012). A potential explanation for this discrepancy could be that the six Acer species studied here show a relatively narrow range of variation in the ionic effect (from 18 % in A. palmatum to 31 % in A. tataricum) compared with the 2–32 % range across 20 species (Jansen et al. 2011). However, a similar narrow range from 15 % to 23 % was also reported by Nardini et al. (2012). Moreover, the ionic effect of the four Acer species measured by Nardini et al. (2012) were based on stem segments that were ca. 10 cm long, while our measurements were based on a stem segment length with at least 80 % of all vessels intact (i.e. closed). Therefore, direct comparison between this study and Nardini et al. (2012) cannot be made.

Conclusions

This paper demonstrates the ionic effect for six, closely related species within the genus Acer. Although this phenomenon has various implications in the field of plant water relations, the actual relevance in planta and the potential relationships between the ionic effect and plant traits have not been elucidated. Our results confirm the absence of pectic polysaccharides in intervessel pit membranes and the lack of a relation with several pit anatomical traits, which reinforces the need for an alternative hypothesis besides the hydrogel hypothesis to provide a full mechanistic explanation of the ionic effect.

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Contributions by the Authors

M.M.K. and M.S. conducted all immunolabelling work, hydraulic measurements and anatomical observations. M.M.K. and S.J. planned the experiments and observations, and J.P.K. assisted with the immunolabelling. All authors contributed substantially to the writing.

Conflicts of Interest Statement

The authors report that they no conflicts of interest.

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Supporting information

The following additional information is available in the online version of this article —

Table S1. Hydraulic conductance values for six Acer species before and after adding 25 mM KCl to a reference solution. Student’s t-test statistics are given for dependant samples.

Table S2. Anatomical features measured with their acronyms and definitions based on Scholz et al. (2013).

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