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Rhamnogalacturonan-II cross-linking of plant pectins via boron bridges occurs during polysaccharide synthesis and/or secretion

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Keywords: rhamnogalacturonan-II, gel electrophoresis, pectin, boron, radiolabelling, cross-linking, cell wall, *Rosa* sp., *Arabidopsis thaliana*

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Rhamnogalacturonan-II (RG-II), a domain of plant cell wall pectins, is able to cross-link with other RG-II domains through borate diester bridges. Although it is known to affect mechanical properties of the cell wall, the biochemical requirements and lifecycle of this cross-linking remain unclear. We developed a PAGE methodology to allow separation of monomeric and dimeric RG-II and used this to study the dynamics of cross-linking in vitro and in vivo. *Rosa* cells grown in medium with no added boron contained no RG-II dimers, although these re-appeared after addition of boron to the medium. However, other *Rosa* cultures which were unable to synthesize new polysaccharides did not show dimer formation. We conclude that RG-II normally becomes cross-linked intraplasmically or during secretion, but not post-secretion.

Boron (B), available in soil as soluble boric acid, is an agriculturally important element for which plants have an absolute requirement. There is a narrow range of optimal concentrations and B deficiency or excess in soil are both problematic, with a range of symptoms such as shortened roots and stems, death of growing-points or roughening of the epidermis.

Early investigations into the effects of B deficiency revealed strong effects on the mechanical properties of plants, with tissues often feeling ‘brittle’, suggesting an important role for B in the structure of plant cell walls. Further studies found a correlation with pectin content, and it was hypothesized that pectic apirose residues, found in the rhamnogalacturonan-II (RG-II) component of pectin, bind B, resulting in the ability to form RG-II dimers cross-linked through B (Fig. 1A).

We decided to investigate the formation of RG-II–(B–)–RG-II bridges and developed a new technique for the separation of RG-II monomers and dimers. Using standard gel electrophoresis equipment, we successfully separated these two compounds (which have similar charge:mass ratio) thanks to their difference in size (~5 and 10 kDa respectively), as is performed for protein SDS–PAGE or oligosaccharide PAGE (Fig. 1B). This technique also allows fluorographic visualization of radioactive products, which were produced by radio-labelling RG-II preparations with NaB³H₄. We demonstrated how this technique allows the rapid monitoring of cross-linking by successfully monomerising dimers of RG-II in vitro (by lowering pH with the addition of 0.1 M HCl) and dimerizing monomers (by adding 0.1–1.0 mM H₂BO₃, a process which was promoted by the addition of Pb²⁺).

In order to study this cross-linking in vivo, we attempted to grow *Rosa, Arabidopsis* and *Spinacia* cell-suspension cultures with reduced H₂BO₃ concentrations in their respective media. The *Arabidopsis* and *Spinacia* cells did not survive in these conditions, but the *Rosa* cells continued to grow (and have been growing for > 2 y). After 8 wk in media with no added boron, only monomeric RG-II was detectable through gel electrophoresis. Re-addition...
of H$_3$BO$_3$ to the medium at the routine concentration (3.3 µM) led to the formation of small amounts of dimeric RG-II after 1 h, with the proportion increasing over 24 h. Interestingly, the quantities of monomeric RG-II did not decrease, suggesting that pre-formed monomeric domains were unable to dimerize and only newly formed RG-II formed dimers. To further test this hypothesis, we applied treatments (carbon starvation, respiratory inhibitors, anaerobiosis, freezing or boiling) to Rosa cells which decrease or prevent de-novo polysaccharide synthesis before re-supplying H$_3$BO$_3$. In all cases, negligible RG-II dimer formation was observed. Importantly, our data also showed that although boron bridges are important for the development of a cell, they are not essential for retaining pectin in the cell wall. Washing the zero-boron cells with Na$_2$CO$_3$ did not remove the pectins demonstrating they were an integral component of the cell wall. This is likely to be due to cross-linking with other cell wall components, possibly through Ca$^{2+}$-bridges or glycosidic bonds.$^{17,18}$

The mechanism by which Pb$^{2+}$ promotes cross-linking is not understood$^{16}$ and it is hypothesized that there may be other substances carrying a similar function in vivo. These could include enzymes, boron carriers or cationic RG-II chaperones, any of which may promote dimerization. We added monomeric RG-II and H$_3$BO$_3$ to spent cell culture medium and monitored dimerization by gel electrophoresis. Additionally, we used low concentrations of radiolabelled [3H]RG-II in case excess amounts of RG-II may be unfavorable to dimer formation. In neither case was dimer formation observed, supporting our hypothesis that cross-linking occurs intraprotoplasmically or during secretion. We also conducted in vitro experiments to investigate the basis of B toxicity. Increasing B concentrations up to 2000-fold did not compromise the dimerization in vitro, but it would be of scientific interest to further investigate the toxicity problems by supplying excess B in vivo.

Figure 1. Boron-bridging of RG-II and separation by gel electrophoresis. (A) Schematic representation of boron-bridging of two pectin molecules (B) Gel electrophoresis separation of monomeric and dimeric RG-II. Sample 3 shows separation of oligomers with DP ~6–16 (hexasaccharide to hexadecasaccharide) resulting from the incomplete digestion of homogalacturonan.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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