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Feruloylated Arabinoxylans Are Oxidatively Cross-Linked by Extracellular Maize Peroxidase but Not by Horseradish Peroxidase

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ABSTRACT Covalent cross-linking of soluble extracellular arabinoxylans in living maize cultures, which models the cross-linking of wall-bound arabinoxylans, is due to oxidation of feruloyl esters to oligoferuloyl esters and ethers. The oxidizing system responsible could be H_2O_2/peroxidase, O_2/laccase, or reactive oxygen species acting non-enzymically. To distinguish these possibilities, we studied arabinoxylan cross-linking *in vivo* and *in vitro*. In living cultures, exogenous, soluble, extracellular, feruloylated [*pentosyl*-^{3}H]arabinoxylans underwent cross-linking, beginning abruptly 8 d after sub-culture. Cross-linking was suppressed by iodide, an H_2O_2 scavenger, indicating dependence on endogenous H_2O_2. However, exogenous H_2O_2 did not cause precocious cross-linking, despite the constant presence of endogenous peroxidases, suggesting that younger cultures contained natural cross-linking inhibitors. Dialysed culture-filtrates cross-linked [^{3}H]arabinoxylans *in vitro* only if H_2O_2 was also added, indicating a peroxidase requirement. This cross-linking was highly ionic-strength-dependent. The peroxidases responsible were heat-labile, although relatively heat-stable peroxidases (assayed on *o*-dianisidine) were also present. Surprisingly, added horseradish peroxidase, even after heat-denaturation, blocked the arabinoxylan-cross-linking action of maize peroxidases, suggesting that the horseradish protein was a competing substrate for [^{3}H]arabinoxylan coupling. In conclusion, we show for the first time that cross-linking of extracellular arabinoxylan in living maize cultures is an action of apoplastic peroxidases, some of whose unusual properties we report.

Key words: Cell wall; cross-links; phenolics; ferulate; peroxidase; soluble extracellular polysaccharides; *Zea mays* L.

INTRODUCTION

Primary cell walls in the Poaceae (grasses and cereals) and their close relatives possess certain unique features, being low in pectins (~5%), high in arabinoxylans (~30%), and relatively high in non-lignin phenolics (0.5–1.5% dry weight) (Harris et al., 1997; Fry, 2000; Fincher, 2009). One specific phenolic compound, ferulic acid, is attached to Poaceae arabinoxylans as a side-chain, and throughout this paper, the term 'arabinoxylan' implies 'feruloylated arabinoxylan', unless otherwise stated. It has been speculated that feruloyl ester groups on adjacent arabinoxylan chains become oxidatively coupled during cell-wall development *in vivo*, thus forming inter-polymeric covalent bonds that cross-link these chains and tighten the cell wall, and that consequently feruloyl-bridged arabinoxylans perform a role in the Poaceae similar to that of calcium- or borate-bridged pectins in plants generally (Hatfield et al., 1999; Pérez et al., 2003; O'Neill et al., 2004; Proseus and Boyer, 2008).

In studies of the biology of polysaccharide cross-linking, it is essential to distinguish the cross-linking reactions that may occur *in vivo* from those that can readily be demonstrated to occur under artificial conditions *in vitro*. Inter-polymeric cross-linking of feruloyl-polysaccharides can be achieved *in vitro*. For example, galactomannans, artificially esterified with ferulic acid, have been shown to gel when exposed to oxidizing agents, and subsequently to release diferulates (also known as dehydrodiferulates) when saponified (Geissmann

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and Neukom, 1971). Feruloylated pectins can likewise be cross-linked *in vitro* (Rombouts and Thibault, 1986; Guillon and Thibault, 1990; Micard and Thibault, 1999), and feruloylated arabinoxylans isolated from wheat flour and wheat bran have been shown to behave in a similar way on treatment with peroxidase + H_2O_2 (Schooneveld-Bergmans et al., 1999).

Various isomers of diferulic acid have been obtained by alkali hydrolysis of cell walls and from *in-vitro* cross-linked feruloyl-polysaccharides (Markwalder and Neukom, 1976; Harris and Hartley, 1980; Hartley and Harris, 1981; Ralph et al., 1994; Brett et al., 1999; Waldron et al., 1996; Bunzel et al., 2004, 2005), and may contribute to the natural cross-linking of arabinoxylans *in vivo*. The diferulate:ferulate ratio was shown to increase when isolated maize cell wall preparations containing bound peroxidase were exposed *in vitro* to dilute hydrogen peroxide (Grabber et al., 1995).

There is some evidence that the diferulates released by alkali from cell walls derive from feruloyl arabinoxylans: for example, a 5,5′-di-(feruloyl-arabinosyl-xylobiose) was isolated from maize bran after Driselase digestion (Ishii, 1991). Furthermore, 5,5′-di-(feruloyl-arabinose) (Saulnier et al., 1999) and 8-*O*-4-di-(feruloyl arabinose) (Allerdings et al., 2005) have been isolated after partial acid hydrolysis of maize bran arabinoxylans. As yet, no oligosaccharides linked to other diferulate isomers, or to larger oxidation products, have been fully characterized.

Although diferulates may contribute to polysaccharide cross-linking, ferulate-products larger than dimers (oligoferulates) often predominate *in vivo* (Fry, 1984; Fry et al., 2000) and *in vitro* (Ward et al., 2001). As well as their being quantitatively predominant, it is also more likely that the larger coupling products could act as real (= inter-polysaccharide) cross-links rather than forming intra-polysaccharide loops* (Fry, 2004a). Therefore, assays of dimers alone may seriously underestimate the degree of feruloyl-polysaccharide cross-linking (if oligoferulates predominate) or overestimate it (if diferulates form mainly intra-polysaccharide loops). Certain specific isomers of triferulic acid (Bunzel et al., 2004, 2005, 2006; Funk et al., 2005; Rouau et al., 2003) and tetraferulic acid (Bunzel et al., 2006) have been isolated and structurally elucidated from primary cell walls in grass and cereal species, particularly from maize bran.

A more general approach for detecting, as a group, total coupling products including those larger than dimers is to feed the cells with [^{14}C]cinnamate, a precursor of ferulate; *all* ferulate-derived substances can then be detected quantitatively, thanks to their radioactivity, whether or not their physicochemical properties (susceptibility to hydrolysis, solubility, chromatographic behaviour, etc.) could have been predicted (Fry, 1984; Fry et al., 2000; Lindsay and Fry, 2008; Burr and Fry, 2009). In maize cell cultures fed with [^{14}C]cinnamate, inhibition of peroxidase action by treatment with iodide, dithiothreitol (DTT), or cysteine caused an increased proportion of [^{14}C]feruloyl-arabinoxylans to be released into the medium, supporting the hypothesis that peroxidase action is responsible for polysaccharide cross-linking in the cell wall *in vivo* (Lindsay and Fry, 2008).

During the progression of maize cell cultures through the culture cycle, the site of diferulate production shifts from the protoplasm to the cell wall (Fry et al., 2000). In younger cultures, pre-cross-linked feruloyl-arabinoxylans are proposed to be secreted into the apoplast as large coagula, which would hydrogen-bond only loosely to the cellulose microfibrils already present in the cell wall, thus maintaining a high extensibility in the growing cell wall. In older cultures, in contrast, the feruloyl-arabinoxylans are secreted into the cell wall as individual, non-cross-linked polysaccharide chains, which would not only form extensive hydrogen bonds with the cellulose, but would also be capable of subsequently forming inter-arabinoxylan covalent bonds via diferulates and oligoferulates, consequently clamping the microfibrils and transforming the cell wall into a less extensible network.

If oxidative coupling occurs between feruloyl groups on adjacent polysaccharide chains *in vivo*, producing a cross-linked network, the effective relative molecular mass (M_r) of the polysaccharide is expected to increase considerably. However, the cell wall polysaccharides, being already hydrogen-bonded into a dense network, are insoluble, and therefore recalcitrant to M_r measurements. Solubilization of wall-bound arabinoxylans is only possible by (pre-) treatments, such as with aqueous alkali or hydroxylamine (Mares and Stone, 1973) or methanolic sodium methoxide (Morrison, 1977), which are likely to cleave the ester-based cross-links under investigation. Fortunately, maize cell-suspension cultures slough some of their wall-related polysaccharides into the culture medium, where, since they remain soluble, they provide a convenient model system for studying polysaccharide cross-linking *in vivo*. Indeed, when cultured maize cells were fed [^{3}H]arabinose, the subsequently released radiolabelled soluble extracellular polysaccharides (mainly feruloylated arabinoxylans) were shown to undergo an abrupt size increase from ~1 to >17 MDa at a time-point between 8 and 11 d into the culture cycle (Kerr and Fry, 2003). This increase was delayed by exogenous sinapic acid or chlorogenic acid, both of which are low-M_r substrates competing with the feruloyl residues of arabinoxylans to be oxidatively coupled (Kerr and Fry, 2004). Consequently, the feruloyl side-chains of arabinoxylans

* The suggestion that diferulates can potentially form intra-polysaccharide loops has been debated. Hatfield and Ralph (1999) modelled *in silico* the oxidative coupling of an oligosaccharide of xylose [β-(1→4)-Xyl_{16}] that carried two feruloyl–arabinose (Fer–Ara) groups variously spaced along this very short backbone. They concluded that, in most such structures, intra-chain looping was sterically implausible. However, the widest spacing tested was with the Fer–Ara groups on the n^{th} and $(n+5)^{th}$ xylose residues—a very short span compared with real cell-wall arabinoxylans, which often have a backbone of several thousand xylose residues. Such a polymer backbone (chain length typically ~2 μm) is ~10× longer than the diameter of a Golgi vesicle, into which the polysaccharide had evidently fitted prior to secretion. This implies that *in vivo*, the polysaccharide chain must be capable of coiling, such that widely spaced Fer–Ara residues (located on, say, the n^{th} and $(n+2000)^{th}$ xylose residues) could readily come into close contact, enabling diferulates to form intra-polysaccharide loops. We therefore do not accept Hatfield and Ralph's argument against intra-chain coupling.