Brief Communication

A dominant negative approach to reduce xylan in plants

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The most abundant and renewable source of carbohydrates is found in the lignocellulosic biomass of plants, contained in the cell walls surrounding plant cells. However, the plant cell wall has evolved to be rigid and nearly impenetrable, making the conversion of plant biomass to simple sugars a laborious and expensive endeavour. After cellulose, a class of polysaccharides called hemicelluloses make up the majority of the carbohydrate content of plant biomass. The β-(1,4)-linked xylose homopolymer xylan is the most abundant hemicellulose in secondary cell walls of angiosperms (Scheller and Ulvskov, 2010). Many genes with unique, if not well understood, roles in xylan biosynthesis have been identified. Members of two glycosyltransferase (GT) families are required for synthesis of the xylan backbone: IRREGULAR XYLEM 9 (IRX9) and IRX14 from GT43 and IRX10 from GT47 (Brown et al., 2009; Rennie and Scheller, 2014; Peña et al., 2007). In Arabidopsis thaliana, each protein has a single homolog with partial functional redundancy: IRX9L, IRX14L and IRX10L, respectively. Due to redundancy, not all the single mutants have a strong phenotype, but in general, mutants in these genes and their paralogs have reduced xylan content, shorter xylan chains and lower endogenous xylan synthase activity (Brown et al., 2009; Rennie and Scheller, 2014; Peña et al., 2007). The reduced xylan often results in thinner and weaker secondary cell walls, leading to the irregular collapsed xylem vessels for which the genes are named. Only IRX10 and IRX10L have been demonstrated to have β-(1,4)-xyllosyltransferase activity in vitro (Urbanowicz et al., 2014; Jensen et al., 2014). The lack of evidence for their catalytic roles has led to the hypothesis of a xylan synthase complex wherein IRX9L and/or IRX14L play primarily structural roles and IRX10(L) is responsible for elongation of the xylan backbone.

While its interaction with cellulose makes xylan a critical part of the structural integrity of the cell wall, it also makes xylan a contributor to the recalcitrance of plant biomass to enzymatic degradation. An additional downstream obstacle presented by xylan is the large amount of pentoses and acetyl groups released during its enzymatic breakdown. Pentoses cannot be efficiently metabolized and may inhibit the fermentation of hexoses like glucose in commonly used microbial platforms, for example Saccharomyces, and acetate is generally detrimental to their growth (Young et al., 2010). Therefore, reducing the amount of xylan in the plant secondary cell wall is an important goal in the development of biofuel crops. The ideal strategy would be to reduce the amount of xylan in a wild-type plant since few knockout mutant lines of relevant biofuel crops exist. Knockout mutants could be generated using genome editing, but in many cases, multiple genes would have to be targeted to achieve the desired phenotype; for example, there are four homologs of IRX10 in sorghum and seven in rice.

Here, we report the dominant suppression of xylan biosynthesis in wild-type A. thaliana via the overexpression of two mutated forms of IRX10. The amino acid sequence of AtrRX10 was aligned with those of the closest homologs in A. thaliana and a range of plant species (Oryza sativa, Populus trichocarpa, Brachypodium distachyon, Plantago ovata, Physcomitrella patens), as well as the Homo sapiens homolog Exostosin1 (EXT1) (Figure 1a). The degree of conservation of amino acid residues was used to infer potential roles of specific residues in catalysis and identify targets for mutation. One amino acid in particular, Glycine-283, is conserved in all sequences and is known to be important for the function of EXT1 in humans (Duncan et al., 2001). G283 is clustered with three other residues that are fully conserved across land plants: F277, C278 and E293. The proximity of these residues to G283 and their large and/or charged functional groups made these residues attractive additional targets. F277 and C278 were mutated to alanine, and G283 was mutated to aspartate. E293 was mutated to glutamine to maintain the size but remove the charge of the functional group. A fifth conserved residue in a different portion of the protein, H146, was mutated to aspartate. Each single amino acid substituted version of the IRX10 coding sequence, as well as the native sequence, was then used to create individual transgenic A. thaliana lines with a wild-type Col-0 background. The coding sequences were expressed with a C-terminal FLAG epitope fusion under the strong, constitutive cauliflower mosaic virus 35S promoter. The first generation of transgenic plants carrying the F277A, G283D and E293Q forms of IRX10 displayed significantly reduced height, while lines expressing the H146D and C277A mutants more closely resembled the empty vector control (EVC) (Figure 1b). Dwarfed stature is an early indication that xylan biosynthesis may be suppressed. Immunoblotting using anti-FLAG antibody served to confirm that expression in individual transgenic lines was be detectable in a crude protein extract. However, expression in all IRX10 and G283D lines was detectable once proteins from the stem were solubilized and enriched via anti-FLAG immunoprecipitation (Figure 1c). While the F277A lines displayed a strong phenotype, we could not confirm expression of the mutated IRX10. The plants were not further analysed, since the phenotype...
Figure 1  (a) Sequence alignment of IRX10 (At1g27440) homologs: *A. thaliana* (IRX10-L, AT5G61840), *P. trichocarpa* (XP_002297880.1, POPTR_0001s12940g), *O. sativa* (Os01g0926700), *B. distachyon* (BRAD1_2g59410), *P. ovata* (IRX10_6, APW77260.1), *P. patens* (XP_001753186) and *H. sapiens* (EXT1, AAB62718.1, 35% id.). (b) Representative plants from the T1 generation. EVC, empty vector control; IRX10, plant transformed with unmutated IRX10. (c) Immunoblot using anti-FLAG antibody showing transgene expression from T2 lines. (d) Alcohol-insoluble residue (AIR) prepared from 6 cm of basal inflorescence stem of fully senesced plants as previously described. AIR samples (1–3 mg) were hydrolysed with 2M trifluoroacetic acid for 1 h at 120°C, lyophilized, resuspended in water and centrifuged at 20,000 g for 10 min. The supernatant was analysed by high-performance anion exchange chromatography on an ICS 5000 instrument (Thermo Fisher) using a CarboPacTM PA20 column (3 × 150 mm, Thermo Fisher). Bars show average ± SD (n = 3–5). P-values indicate significance of difference in xylose content compared to EVC. (e) Immunofluorescence labelling of stem cross sections (left panels, scale bars 100 μm) with the xylan-specific LM10 antibody (PlantProbes, Leeds, UK), and toluidine blue O-stained cross sections (right panels, scale bars 25 μm). The basal regions of inflorescence stems (3 cm above the rosette) were collected from 10-week-old plants and fixed overnight at 4°C in 4% paraformaldehyde, 50 mM PIPES, 5 mM EGTA, 5 mM MgSO4, pH 6.9. The stems were embedded in 7% agarose, and 100-μm sections were made using a Leica VT1000S vibratome. For immunolabelling, sections were washed three times with phosphate-buffered saline (PBS) pH 7 and incubated overnight with a 10-fold dilution of LM10 antibody in PBS. The sections were then incubated with fluorescein isothiocyanate-conjugated goat anti-rat secondary antibodies for 1 h and imaged with a Zeiss LSM710 confocal microscope with ZEN2010 software (Zeiss). Phloem (ph) and xylem (xy) are labelled with arrows and arrowheads indicating irregular and normal vessels, respectively. (f) Xylan synthase activity in transgenic plants. All reactions were performed in a total of 25 μL containing 10 mM MnCl2, 1% (v/v) Triton X-100 and 50 mM MES, pH 6.5. The assays included 2 μg ANTS-labelled xylohexaose, 200 μM UDP-Xyl and microsomal membranes (50 μg total protein). Reactions were incubated for 2 h at 30°C, terminated by heating (100°C, 3 min) and centrifuged at 10,000 g for 10 min. Supernatants (15 μL) were mixed with 15 μL 3 M urea, and the samples (5 μL) were analysed by separation on large format Tritis-borate acrylamide gel prepared as described elsewhere and electrophoresed at 200 V for 30 min followed by 1000 V for 1.5 h. The gels were visualized using a G-box (Syngene, www.syngene.com) with UV detection filter and UV tubes (365 nm emission). Band intensities for Xyl7 and Xyl8 were detected and quantified automatically with GeneTools (Syngene). The sum of the two bands was used as a measurement for XylT activity. Significant differences from EVC are indicated (P < 0.01, ANOVA and Dunnett's multiple comparison test).
could be due to co-suppression of IRX10 with the transgene. When the monosaccharide composition of the stem was analysed, each of the mutant IRX10 overexpressors demonstrated varying degrees of xylose reduction, with the G283D and E293Q mutants being the most significant (Figure 1d). The xylose content of plants overexpressing the native form of IRX10 was comparable to EVC.

The effects of the drastically reduced xylose content in G283D and E293Q T2 stems were further investigated with xylan immunolabelling and toluidine blue O staining of transverse sections of the basal stem (Figure 1e). The xylem vessels of EVC plants and the native IRX10 overexpressors are characteristically large and round in shape with thick secondary cell walls. The xylem vessels of E293Q overexpressors, in contrast, are smaller and more irregular in shape with significantly thinner secondary cell walls. The G283D overexpressors exhibit a less pronounced irregular vessel phenotype despite the thin secondary cell walls and an even greater reduction in xylose than the E293Q overexpressors.

This work reveals that there are at least two amino acid residues, G283 and E293, which are critically important in the xylan biosynthesis activity of IRX10. The fact that overexpressing a mutated form of IRX10 is capable of disrupting the activity of the native IRX10 is consistent with the existence of a xylan synthase complex. The more abundant IRX10 mutant protein is apparently able to displace the functional IRX10 in the complex, thereby abrogating xylan biosynthetic activity. We do not have a method to directly determine the amount of native IRX10 and IRX10L in the plants, but xylan synthase activity assay with microsomes from stems of the transgenic plants confirmed a lower activity in the G283D and E293Q plants (Figure 1f). The proteins that IRX10 and IRX10L interact directly with in a biosynthetic complex have not been unambiguously identified, but there is significant evidence that IRX9(L) and IRX14(L) are part of the complex. It is conceivable that additional proteins are also part of the complex (Jiang et al., 2016).

From a practical perspective, the findings reported here readily suggest a method to improve biomass composition. Down-regulation of xylan biosynthesis in bioenergy crops is desirable because an increased hexose/pentose ratio is advantageous for the downstream processing of biomass (Young et al., 2010). Obviously, in the practical implementation, down-regulation must be done in a way that does not cause a yield reduction. Prior work has demonstrated that when xylan is specifically reduced in interfascicular fibre cells, while maintained in vessels, the resulting plants have a large increase in hexose/pentose ratio while not showing any reduction in growth (Petersen, 2012). Hence, the expression of the mutated IRX10 forms described here under strong fibre-specific promoters would be a potential way to engineer plants with the desired properties. Alternatively, the expression of the mutated forms in vessels could be eliminated in various ways. Considering the degree of protein sequence conservation, our approach could be easily adapted to a variety of relevant crop species. The biosynthetic pathway targeted in this paper is xylan biosynthesis. However, the same kind of dominant negative approach can be used in most cases that involve protein complexes. Dominant negative protein variants could be used as a biotechnology tool in other systems to reduce biosynthetic activity, especially where genetic redundancy makes genome editing or mutagenesis impractical.

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Conflict of interests

AGB and HVS are inventors on a patent application related to the work described here.

Author contributions

AGB and HVS designed this study and wrote the manuscript. DB analysed monosaccharide composition and AGB conducted all other experiments.

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