Arabidopsis GELP7 functions as a plasma membrane-localized acetyl xylan esterase, and its overexpression improves saccharification efficiency

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Received: 25 August 2021 / Accepted: 12 April 2022 / Published online: 17 May 2022
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Abstract
Acetyl substitution on the xylan chain is critical for stable interaction with cellulose and other cell wall polymers in the secondary cell wall. Xylan acetylation pattern is governed by Golgi and extracellular localized acetyl xylan esterase (AXE). We investigated the role of Arabidopsis clade Id from the GDSL esterase/lipase or GELP family in polysaccharide deacetylation. The investigation of the AtGELP7 T-DNA mutant line showed a decrease in stem esterase activity and an increase in stem acetyl content. We further generated overexpressor AtGELP7 transgenic lines, and these lines showed an increase in AXE activity and a decrease in xylan acetylation compared to wild-type plants. Therefore, we have named this enzyme as AtAXE1. The subcellular localization and immunoblot studies showed that the AtAXE1 enzyme is secreted out, associated with the plasma membrane and involved in xylan de-esterification post-synthesis. The cellulose digestibility was improved in AtAXE1 overexpressor lines without pre-treatment, after alkali and xylanases pre-treatment. Furthermore, we have also established that the AtGELP7 gene is upregulated in the overexpressor line of AtMYB46, a secondary cell wall specific transcription factor. This transcriptional regulation can drive AtGELP7 or AtAXE1 to perform de-esterification of xylan in a tissue-specific manner. Overall, these data suggest that AtGELP7 overexpression in Arabidopsis reduces xylan acetylation and improves digestibility properties of polysaccharides of stem lignocellulosic biomass.

Keymesage
Identification and characterisation of Arabidopsis acetyl xylan esterase from GELP family

Keywords  Acetyl xylan esterase · Plant cell wall · Deacetylation · Saccharification · GELP7 · Arabidopsis · GDSL esterase

Introduction
The plant cell wall, a complex and dynamic meshwork made up of polysaccharides, mainly cellulose, hemicellulose (xylan, xyloglucan and mannan) and pectin with lignin impregnation. It plays a pivotal role in plant growth, development, and maturation because of its flexible and rigid nature. Also, the cell wall is rich in cellulosic, non-cellulosic polysaccharides and polyphenols, a major source for the production of biofuels and other bio-based materials (Donev et al. 2018). But the complex and intricate cell wall structure is a major hurdle during the processing of plant biomass to biofuel. Enzymatic hydrolysis of the plant cell wall is significantly hindered by the presence of acetyl groups substituted on hemicellulosic and pectic polysaccharides. Also, enzymatic deacetylation of biomass leads an increase in acetate
level in the fermentation medium that interferes with microbial growth by lowering the pH of the medium (Pawar et al. 2013). One way to deal with this problem is modulating polysaccharide acetylation by genetic manipulation of cell wall acetylation pathways in plants. In planta reduction of acetyl content exposes polysaccharides during the saccharification process. Therefore, polysaccharide acetyl esterases can be utilized to finetune the acetate level of the cell wall. In planta overexpression of fungal AXE in Arabidopsis and poplar resulted in enhanced cellulose and xylan hydrolysis. Also, decreasing xylan acetylation increases the fermentation efficiency of the Arabidopsis plant cell wall because of less accumulation of acetic acid (Pawar et al. 2016, 2017; Wang et al. 2020).

Few pectin acetyl esterase (PAE) and acetyl xylan esterase (AXE) are identified in plants, but their role in plant cell wall organization and recycling is unclear. PAEs belong to the carbohydrate esterase (CE) family, a large family found in higher plants. Heterologous expression of poplar PtPAE1 in tobacco leads to abnormalities in floral organs (Gou et al. 2012). And overexpression of mung bean PAE in potato tubers increases the stiffness of the plant cell wall (Orfila et al. 2012). Mutation in Arabidopsis AtPAE8 and AtPAE9 reduces pectin acetylation content and inflorescence stem size (de Souza et al. 2014). Recently, AXE, which belongs to the GDSL esterase/lipase or GELP family, have been identified and characterized in rice named as RICE MALE STERILE 2 (RMS2) are involved in ethylene signaling and lipid homeostasis, respectively (Zhao et al. 2020a, b). Furthermore, IPE2 (IRREGULAR POLLEN EXINE 2) is a GDSL lipase in maize which is essential for male fertility (Huo et al. 2020). Several Arabidopsis GELP members have recently shown key roles in suberin polymerization and degradation (Ursache et al. 2021). In summary, only a few members from the GELP family are characterized, and they function in lipid, glucosinolate and cell wall metabolism by showing either lipase or esterase activity. Identification of their role in xylan de-esterification will enable us to understand xylan biosynthesis, modification and recycling. And those esterases can be effectively utilized to alter plant biomass for generation of value-added products.

In this project, ArGELP7 or AtIG28580 is identified as acetyl xylan esterase (AtAXE1). Transient expression of AtAXE1 in Nicotiana benthamiana revealed that it is esterase but not lipase. Arabidopsis axtaxe1 mutant showed a decrease in esterase activity and increased stem acetyl content suggesting that this could be polysaccharide esterase. Furthermore, overexpressor transgenic lines of AtAXE1 showed a reduction in xylan acetylation. Also, an increase in cellulose and xylan digestibility in AtAXE1 overexpressor lines revealed the potential use of decreasing polysaccharide acetylation to improve lignocellulosic biomass processing.

Results

Gene members of clade Id from GELP family could be involved in xylan acetylation

GDSL esterase/lipase or GELP family is a multigene family with diverse role in lipid and cell wall metabolism. Serine (S), Glycine (G), Asparagine (N), and Histidine (H) are conserved in four blocks in all GELP sequences. To
identify acetyl xylan esterase (AXE) from the Arabidopsis GELP family, the sequences were fetched from (https://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/) using query SGNH. A total of 103 amino acid sequences from Arabidopsis and two AXEs (BS1 and DARX1) from rice were retrieved. Neighbor-Joining tree revealed that GELP family is divided into two main clades (I and II) (Fig.S1). Clade I is divided into ten subclades (Ia to Ij). BS1 is clubbed in clade Id, and the GDSL domain is conserved in all the sequences of clade Id. These sequences were used for phylogenetic and other bioinformatic studies. All the amino acid sequences from GELP family had GDS domain conserved at N-terminal (Fig.S2). Also, the sequences from clade Id showed sequence identity from 28 to 42% with rice BS1 (Fig.S3). All the members from clade Id had either TAP-like or acetylhydrolase domain and the protein containing either of these domains show esterase activity. This bioinformatics analysis suggested that this clade could be involved in xylan deacetylation.

**AtGELP7 is upregulated in overexpressed MYB46 transgenic lines**

Xylan is one of the major secondary cell wall polysaccharides in dicot plants. MYB46 is a direct target of xylan biosynthetic genes. TBL29, RWA, and ATP-citrate lyase (ACL) are functioned in xylan acetylation biosynthesis and upregulated in AtSND1 overexpressor line through feedforward loop by interacting with MYB46 (Lee et al. 2011; Yuan et al. 2013; Zhong et al. 2020). Our hypothesis was GELP family members are involved in xylan acetylation and its expression is regulated by MYB46. Therefore, we generated five independent Arabidopsis transgenic lines overexpressing MYB46. All the five lines showed more expression than wild type plants (Fig. 1A). Overexpression (OE)-2 was a highly expressed line among all with five-fold expression, followed by OE-3 and OE-5 with approximately three-fold expression. All MYB46 OE lines look like wild type plants (Fig. 1B). We found that PAL2 expression was elevated in OE-2 and OE-5 lines (Fig. 1C) which was correlating with previous findings (Yuan et al. 2013). We tested the expression of selected genes from clade Id and Ic. We did not see any change in At2G27360 (AtGELP53), At1G28670 (AtGELP14), At1G28570 (AtGELP6), At5G45910 (AtGELP10), and At1G09390 (AtGELP2). But we found that At1G28580 or AtGELP7 was 55-fold higher in both MYB46 OE lines as compared to wild type (Fig. 1D). This suggests that AtGELP7 is likely to function in the secondary cell wall or xylan biosynthesis.

**Transient expression in Nicotiana confirms that AtGELP7 or AtAXE1 is a plasma membrane-localized esterase**

AtGELP7 will be referred as AtAXE1, as explained below. AtAXE1 was cloned into an expression vector having 35S constitutive promoter and transiently expressed in Nicotiana benthamiana. The members from the Arabidopsis GELP family showed both esterase and lipase activity (Lai et al. 2017). The soluble and wall-bound protein was extracted from transiently expressed AtAXE1 leaf tissue. 4-nitrophenyl acetate was used as a substrate and wall localized acetyl xylan esterase from Aspergillus niger (AnAXE1) as a positive control for esterase activity (Pawar et al. 2016). As expected, AnAXE1 showed an increase in wall-bound esterase activity (Fig. 2A). Both soluble and wall-bound protein fractions showed an increase in esterase activity when AtAXE1 was expressed in Nicotiana leaves (Fig. 2A). Some of the Arabidopsis GELP family members exhibited lipase activity (Huo et al. 2020; Zhao et al. 2020a; Ursache et al. 2021). Therefore, lipase activity was tested using 4-nitrophenyl palmitate as a substrate and lipase activity in AtAXE1 infiltrated leaf was the same as that of uninfiltrated Nicotiana leaves (Fig. 2B). This signifies that AtAXE1 doesn’t show lipase activity on the above synthetic substrate.

To check the subcellular localization of AtAXE1 in the plant cell, AtAXE1 was fused with an N-terminal GFP tag and expressed in Nicotiana leaves along with RFP tagged Plasma Membrane Intrinsic Protein 2A (PIP 2A) marker. AtAXE1 GFP signal was observed in the membrane (Fig. 2C). Co-localization using RFP plasma membrane marker confirmed that it is in the plasma membrane. To distinguish GFP localization further, Nicotiana leaf epidermal cells expressing both GFP-AtAXE1 and RFP were plasmolyzed with 30% glycerol. The colocalization of both RFP and GFP signals was observed after plasmolysis. This study was further confirmed by performing co-localization experiments in Arabidopsis, which revealed its localization in the plasma membrane (Fig.S4). Bioinformatic analysis showed At1G28580 or AtAXE1 has a signal peptide and two transmembrane domains. (Fig.S5 and Fig.S6). To confirm AtAXE1 is attached to the membrane, we extracted total (TP), soluble (SP), and wall-bound (WB) protein fractions from Nicotiana infiltrated GFP-AtAXE1 and wild type leaves which can be visualized on Ponceau S stained blot (Fig. 2D). The immunoblotting of these fractions revealed accumulation of GFP in TP and WB fraction but not in SP (Fig. 2E). Altogether this data indicates that AtAXE1 is a plasma membrane-localized esterase.
T-DNA mutant of AtAXE1 exhibited a decrease in esterase activity and an increase in polysaccharide acetylation.

To further test the function of AtAXE1, the SALK_030805 T-DNA insertion line was obtained from Arabidopsis Biological Research Centre (ABRC) as no other mutant allele was available. The T-DNA insertion was found at the 3’ region of AtAXE1 gene, suggesting that it could be knockdown mutant (Fig. 3A). Homozygous mutants were isolated by genotyping, which morphologically resemble the wild-type plant (Fig. 3B and C). The RNA level expression of AtAXE1 gene was checked in leaf and stem tissues of homozygous ataxe1 mutant by RT-PCR which showed reduction in expression of both the tissues. Mutant stem and leaf showed 82% and 37% decrease in expression in comparison with wild type plants respectively (Fig. 3D and E, Fig.S7A). This suggests that the SALK_030805 line is a knockdown mutant of AtAXE1. Specific esterase activity was found to be increased in mutant leaf tissue by 12% in comparison with wild type plants respectively (Fig. 3F and G). To further check relative acetylation level, dried leaf and stem powder was subjected to Fourier Transform Infrared Spectroscopy (FTIR). Comparison of wild type and mutant powder suggested an increase in acetyl linkages at 1240 cm⁻¹ in mutant plant stem tissue but not in leaf tissue (Fig.S7D and Fig.S7E). The wave-number 1240 cm⁻¹ corresponds to C–O linkage in acetyl groups (Gorzsás et al. 2011). These results signify a relative increase in acetylation of the stem tissue. Decrease in esterase activity led to a 12% increase in polysaccharide acetylation in ataxe1 mutant.

Analysis of Arabidopsis 35S::AtAXE1 transgenic lines further confirms that it is a wall-bound esterase

AtAXE1 is ubiquitously expressed in all the tissues but highly in inflorescence stem, fresh rosette leaf and senescence leaf (Fig. 4A). To validate the function of AtAXE1 further, Arabidopsis transgenic lines overexpressing AtAXE1 were generated, and three independent lines, i.e., 2b, 3b, and 5b, were analyzed for further studies. Wild-type and transgenic plants had no substantial morphological distinction (Fig. 4B). Modifying polysaccharide acetylation

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Representation of MYB46 overexpressor line and expression of Arabidopsis GELPs in 35S::MYB46 over-expressing lines. A Expression of MYB46 in 5 independent MYB46 transgenic lines. B Representative picture of wild type and MYB46 transgenic line. C Expression of PAL2, involved in lignin biosynthetic pathway and D Expression of AtGELPs in 2 highly over-expressing MYB46 transgenic lines, MYB46 OE-2 and MYB46 OE-3. Data represents mean ± SE, n = 3–4 biological replicates, Student’s t-test at **p ≤ 0.05, *p ≤ 0.1
Fig. 2 Temporal expression of AtAXE1 in Nicotiana leaves. Infiltrated Nicotiana benthamiana leaves with Agrobacterium carrying 35S::AtAXE1 and performed (A) esterase activity using 4-nitrophenyl acetate as a substrate, and (B) lipase activity using 4-nitrophenyl palmitate as a substrate, and product 4-nitrophenol was analysed in both assays by spectrophotometer at 400 nm. C AtAXE1 was fused with N-terminal GFP and coinfiltrated with plasma membrane RFP marker in Nicotiana leaf epidermal cells. The pictures were captured before and after plasmolysis with 30% glycerol after 2 days of infiltration. R represents Pearson’s Correlation. D Western blot stained with Ponceau S showing different protein fractions from Nicotiana i.e., total protein (TP), soluble protein (SP), and wall-bound protein (WB). The blot represents equal protein loading concentration of the samples. E Immunoblot showing abundance of GFP tagged AtAXE1 in WB protein fraction which was probed with anti-GFP antibody. SP and WB protein fractions were probed with anti-GAPDH and anti-V-ATPASE, respectively. The protein molecular weight standards are indicated on the left side in kDa. Bar diagram data represents mean ± SE, n = 3–4 biological replicates, Student’s t-test at **p ≤ 0.05, *p ≤ 0.1
can lead to abnormality in xylem cell morphology. It was checked by staining Arabidopsis inflorescence stem sections using toluidine blue, and no abnormalities were observed in xylem vessel cells (Fig. S8). Overexpression of the AtAXE1 gene was observed in leaf and stem tissue of transgenic lines by RT-PCR where line 5b showed the
highest expression in both tissues in comparison with lines 2b and 3b (Fig. 4C and D). Esterase activity was checked using a synthetic substrate, i.e., para-nitrophenyl acetate. It was significantly increased in both soluble and wall-bound protein fractions from leaf and stem tissues of transgenic plants (Fig. 4E and F).

To elucidate cell wall acetate level of stem and leaf, alcohol insoluble residues (AIR) was subjected to saponification, and acetic acid release was analyzed. The highly expressed transgenic line 5b showed 16%, and 2b, 3b lines showed a 10.5% reduction in leaf acetyl content (Fig. 4G). Stem acetyl content was also reduced in all transgenic lines with an average of 13% (Fig. 4H). These results demonstrated that AtAXE1 is involved in polysaccharide deacetylation.

**Sequential extraction, FT-IR, and enzyme activity analysis revealed that AtAXE1 is acetyl xylan esterase**

To investigate further into more detail whether AtAXE1 acetyl esterase prefers any specific acetylated polysaccharide as a substrate, we sequentially extracted pectin, xyloglucan and xylan in leaf fraction and analyzed the acetylation level. The transgenic line 5b showed increase in acetyl content of pectin (Fig. 5A). The pellet fraction containing xyloglucan and xylan was analyzed and acetyl content was reduced in that fraction of all transgenic lines (Fig. 5B). The pellet fraction was further digested by endoglucanases to remove xyloglucan and acetyl content was decreased in all transgenic lines (Fig. 5C). This confirmed that xylan acetylation is reduced in these transgenic lines.

This was verified by analyzing acetylation level in DMSO rich xylan fraction, and line 2b and 5b showed a decrease by 9% and 21%, respectively, in comparison with wild type (Fig. S9A). To confirm these results, stem powder samples were subjected to FTIR analysis. Spectral analysis showed less abundant 1210–1275 cm⁻¹ (C–O–C) acetyl linkage in transgenic lines in comparison with wild type of both leaf and stem (Fig. 5DE and Fig. S9B). The spectral range 1730–1750 cm⁻¹ corresponding C = O acetyl linkage was also reduced in stem tissues of transgenic plants (Fig. 5D).

We further calculated the ratio of acetyl to xylose linkage, and it was decreased in transgenic lines as compared with wild type (Fig. 5E and Fig. S9C). This data shows the preferential substrate for AtAXE1 is acetylated xylan. Therefore, we checked the esterase activity with acetylated xylan as substrate and found increased esterase activity in both soluble and wall-bound protein fractions in all transgenic lines (2b, 3b and 5b) expressing AtAXE1 (Fig. 5F). In conclusion, sequential extraction, FT-IR and enzyme activity data confirmed that AtAXE1 functions as an acetyl xylan esterase.

Xylanase digested AtAXE1 transgenic lines revealed an increase in xylan digestibility and confirmed that it is involved in xylan deacetylation

To test accessibility of xylanases in transgenic lines, we carried out xylan oligosaccharide mass profiling (OLIMP) analysis where insoluble alcohol residues from stem tissue of transgenic and WT plant was subjected to digestion by GH11 β-endoxylanase that resulted in the release of neutral and acidic xylo-oligosaccharides (XOS). Neutral XOS released ranging from degree of polymerization DP2 to DP6, which are either acetylated or non-acetylated (Fig. 6A, Fig. S10). Acetic XOS ranging from DP3 to DP5 are acetylated and substituted with either glucuronic acid or methyl glucuronic acid (Fig. 6B, Fig. S11). In neutral XOS profile, shorter chain XOS i.e., xylobiose (Xyl2) and diacetylated xylobiose (Xyl2Ac2) were relatively elevated in transgenic lines. Whereas less abundant diacetylated xylotriose (Xyl3Ac2), tetra or penta acetylated xylotetraose were relatively decreased in transgenic lines in comparison with wild-type. Acidic XOS analysis showed similar trend that more abundance of shorter chains GlcA-X3Ac1 and (Me)GlcA-X3Ac1 and less abundance of (Me)GlcA-X4Ac2 and (Me)GlcA-X5Ac3. Generation of more number of shorter chains indicated efficient hydrolysis in AtAXE1 OE lines by xylanases. These results suggest that xylan from transgenic lines was more accessible for GH11 xylanases because of less xylan acetylation in transgenic lines overexpressing AtAXE1. This further confirmed that AtAXE1 acts as an acetyl xylan esterase, and its constitutive expression enhances xylan digestibility.

Improved saccharification efficiency in AtAXE1-overexpressing Arabidopsis transgenic lines

To test the effect of AtAXE1 overexpression on saccharification efficiency of lignocellulosic biomass, alcohol insoluble residues from ball milled dried stem of transgenic and WT plants were subjected to an enzyme cocktail containing cellulase and β-glucosidase and analyzed for the glucose release by GOD-POD assay. Glucose analysis without pretreatment AIR samples showed 27% increase in glucose release in highly overexpressing line 5b than in WT (Fig. 7A). In another experiment, the cell wall powder was pretreated with 0.4 M NaOH, which de-esterifies and dissolves hemicellulosic polysaccharides. Cellulose digestibility was again increased in the 2b, 3b, and 5b lines by 5%, 7% and 12%, respectively (Fig. 7B). We also treated biomass with xylanases, analyzed the digestibility of cellulose, and glucose release was increased by 13% and 9% in line 2b and 5b, respectively, as compared to wild type (Fig. 7C). To further analyze changes in the cell wall because of AtAXE1
overexpression, we measured cellulose, lignin and xylan content because these polymers are major contributing factors for biomass recalitance. The total cellulose and xylose content were similar in wild-type plants and AtAXE1 overexpressor plants (Fig. 7D and Fig. 7E). But the lignin content was reduced in all transgenic lines compared to wild-type plants (Fig. 7F). The overall conclusion from these experiments is that AtAXE1 overexpression increases cellulose digestibility and reduces lignin content.

**Discussion**

The biosynthetic mechanism of xylan acetylation needs attention because of its role in maintaining xylan stability and cell wall assembly (Qaseem and Wu 2020). Golgi localized proteins involved in xylan acetylation, biosynthesis and modification are characterized extensively in Arabidopsis. Post synthetic xylan acetylation modification is hypothesized to be controlled by apoplastic acetyl xylan esterases (AXEs) that are uncharacterized in plants. Here, we have identified and characterized plasma membrane-localized AtGELP7. The functional characterization studies revealed that it is involved in xylan deacetylation. AtGELP7 or AtAXE1 expression is induced in MYB46 overexpression lines, suggesting that AtGELP7 promoter could be direct or indirect target of MYB46. AtAXE1 overexpression decreases xylan acetylation and increases Arabidopsis polysaccharide digestibility with and without pretreatment of lignocellulosic biomass.

Arabidopsis GELP family is a multigene family member, and a few members of this family are characterized which play a role in ethylene signalling, lipid homeostasis, and suberin polymerization (Dong et al. 2016; Lai et al. 2017; Huo et al. 2020; Zhao et al. 2020a, b; Ursache et al. 2021). The two rice Golgi-localized GELPs are characterized, i.e., BS1 and DARX1, which are involved in de-esterification of xylose and arabinose residues, respectively of arabinoxylan (Zhang et al. 2017, 2019). These two esterases play an important role in regulating acetylation patterns in Golgi. The Golgi synthesized acetylated xylan is deposited to the cell wall. There are many known polysaccharide modifiers such as pectin methyltransferase, pectin acetyl esterase, pectate lyase, endoxylanase, endotransglycosylase, endoglucanase which are involved in cell wall remodeling and recycling (Barnes and Anderson 2018). Poplar xylan endotransglycosylase is involved in maintaining tensional stress by finetuning interaction between xylan and cellulose (Derba-Maceluch et al. 2015). It is also known that even chain substitution of acetyl on xylan is necessary for the stable interaction of xylan and cellulose (Grantham et al. 2017). And changing the acetylation level and pattern of substitution destabilizes interaction between xylan-cellulose, might lead to the weaker cell wall. Extracellular AXEs can maintain acetylation levels and can impact cellulose and xylan interactions. Here, we have characterized plasma membrane localized AXE from Clade Id from Arabidopsis GELP family (Fig.S1). Our transient expression studies demonstrated that AtAXE1 does not show any lipase activity, but esterase activity (Fig. 2A and Fig. 2B). Confocal imaging data revealed that it is co-localizing with plasma membrane marker and its localization was further confirmed by plasmolysis and immunoblotting (Fig. 2C, Fig.S4). AtAXE1 contains two transmembrane domain suggesting that may be anchored to membrane. The AtAXE1 attached to GFP was only found in wall bound protein fraction of Nicotiana infiltrated leaves that confirmed AtAXE1 location in cell (Fig. 2D, E).

The AtAXE1 was further characterized by generating transgenic lines overexpressing AtAXE1. The esterase activity was increased in all transgenic lines which was tested using 4-nitrophenyl acetate and acetylated xylan as substrates (Figs. 4F and 5F). Moreover, the acetyl content was reduced in both leaf and stem tissue (Fig. 4G and 4H). The acetyl content in sequentially extracted xylan rich fraction and DMSO xylan fraction was reduced by 18% and 21% in highly expressed transgenic line 5b respectively (Fig. 5C and Fig.S9A). This confirmed that AtAXE1 influences acetyl level on xylan. To further confirm these results, we analyzed XOS released after xylanase digestion and observed generation of shorter chain XOS and less of longer chain XOS by MALDI-TOF-MS in AtAXE1 transgenic lines (Fig. 6). These results were corroborated with our FT-IR data where acetyl to xylose ratio was lower in transgenic lines in comparison with wild type plants (Fig. 5D and 5E). It might be possible that AtAXE1 is active on other polysaccharide like acetylated xylol glucan or mannan as constitutive expression of AXE from Aspergillus niger in Arabidopsis showed decrease in xylol glucan acetylation (Pawar et al. 2016). Also, AXEs from different carbohydrate esterase (CEs) families shows broad substrate specificity i.e., on several acetylated polysaccharides. The detailed biochemical characterization of purified AtAXE1 is necessary using different acetylated
polysaccharide substrates to elucidate its role in plant cell wall organization or degradation.

Additionally, we observed a compensatory increase in pectin acetylation in highly expressed line 5b (Fig. 5A). Altering polysaccharide xylan acetylation led to increase in pectin acetylation and substitution of glucuronic acid on xylan chain (Xiong et al. 2015; Pawar et al. 2016). Our results are consistent with previous findings that cell wall acetylation mechanism is in tight homeostasis control. OE::AtAXE1 transgenic lines did not show any morphological or anatomical phenotype like collapsed xylem vessel, which is commonly observed in dwarf cell wall mutants (Fig. 4B and Fig.S8). Around 20% reduction in xylan acetylation was tolerated by AtAXE1 overexpressor lines as we do not see any effect on plant growth or xylem cell morphology of transgenic lines. Arabidopsis ataxel mutant also showed an increase in stem acetylation by 12% because of less AXE activity (Fig. 3). An increase in stem acetylation led to dwarfism in rice BS1 mutants because of vessel cell spatial patterning but this was not observed in ataxel mutants (Zhang et al. 2017). The probable reason is 12% increase in stem acetylation tolerated by Arabidopsis. Whether more increase in stem acetylation is tolerated by Arabidopsis needs to be investigated in higher order gelp Arabidopsis mutants.

Secondary cell wall transcription machinery is investigated widely in Arabidopsis. MYB46 downstream of SND1 transcription factor which directly regulates expression of secondary cell wall biosynthetic genes (McCarthy et al. 2009). Uregulation of MYB46 enhances lignin, xylan and cellulose biosynthesis by increasing expression of genes in biosynthetic pathways and MYB46 OE lines
accumulate more xylose and lignin. OE of MYB46 and SND1 specifically upregulated xylan acetylation biosynthetic genes such as ATP-citrate lyase (ACL), RWA, and TBL29 (Lee et al. 2011; Yuan et al. 2013; Zhong et al. 2020). Most of the promoter of these genes have Secondary cell wall MYB Responsive Element (SMRE) sites which is target of MYB46. Since our hypothesis was GDSL family might be involved in xylan acetylation, we generated overexpressor MYB46 lines and AtAXE1 expression was increased by 55-fold as compared to wild type plants (Fig. 1). The promoter of AtAXE1 also contains SMRE4 (ACCAACC) and SMRE5 (ACCTAAT), suggesting that MYB46 can bind directly to activate AtAXE1 expression (Zhong and Ye 2012). It cannot be ruled out that MYB46 might be indirectly activating AtAXE1 through another transcription factors. These results indicated that xylan acetylation and deacetylation mechanism is directly or indirectly regulated by SCW transcription factors.

Chemical deacetylation of cell wall polysaccharides using KOH led to increase in sugar yield because it reduces crosslinking between cell wall polymers, but it can negatively affect xylan solubility (Kong et al. 1992). Enzymatic deacetylation prior to cellulose hydrolysis can positively affect both cellulose and xylan digestibility (Zhang et al. 2011). Also, lowering acetyl content improves fermentation efficiency as it balances pH in fermentation medium (Ranatunga et al. 1997). Because of these reasons it was hypothesized that in planta deacetylation is a potential
strategy to improve biomass saccharification and fermentation. Previously we have shown that heterologous expression of *Aspergillus niger* AXE improved polysaccharide digestion with and without pretreatment in Poplar (Pawar et al. 2017). Arabidopsis *AnAXE* lines with 32% decrease in stem acetyl content showed around 19% increase in stem saccharification efficiency with hot water and alkali pretreatment (Pawar et al. 2016). Poplar *AnAXE1* constitutive expression led 26% increase in glucan conversion rate because of 13% reduction in cell wall acetyl content. In this study *AtAXE1* highly expressed transgenic line 5b showed 12% decrease in stem acetyl content (Fig. 4H) and glucose release was increased by 27% and 12% without and with alkali pretreatment in highly expressed transgenic line (Fig. 7A and B). The cellulose digestibility was increased by 9% after xylanase digestion and this suggests that less xylanases concentration in cocktail will be needed in hypoacetylated plants (Fig. 7C). XOS analysis pattern after xylanases digestion also revealed more accessible xylan of all transgenic lines as compared to wild type plants (Fig. 6). This further suggest that *in planta* deacetylation of xylan increases accessibility of both xylanases and cellulases. All these modifications in acetyl content were done after post-synthetic deposition of xylan. Reducing the level of xylan acetylation in Golgi by altering expression of *TBL29* gene does not impact saccharification efficiency in Arabidopsis. This suggests that post-synthetic modulation in acetylation level positively impacts polysaccharide digestibility. One of the possible hypotheses is that Golgi modified acetylated xylan tightly bound cellulose during deposition in Arabidopsis. In vivo xylan labelling techniques are necessary to validate such hypothesis. Our saccharification data corroborates with previous results that post-synthetic xylan deacetylation increases the saccharification efficiency further strengthening our hypothesis that hypoacetylated plants are attractive target for improving lignocellulosic biomass properties.

In summary, we have identified plasma-membrane localized acetyl xylan esterase from GELP family i.e., *AtGELP7* or *AtAXE1*, upregulated in MYB46 OE lines. Increasing *AtAXE1* activity constitutively increases cellulose and xylan digestibility in Arabidopsis inflorescence stem.
Materials and methods

Generation of phylogenetic tree

Arabidopsis 103 amino acid sequences were fetched from superfamily database (https://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/) using SGNH as a query. Rice BS1 (LOC_Os02g15230.1) and DARX1 (LOC_Os05g06720.) sequences were fetched from rice genome annotation project (http://rice.plantbiology.msu.edu/). The phylogenetic tree was generated by MEGA-X tool using Neighbor-Joining method with 250 bootstraps.

Cloning of AtGELP7 and MYB46

AtGELP7 (AT1G28580) and MYB46 (AT5G12870) were amplified using complete attB flanking primers PCWL41, and PCWL42 and partial attB flanking primers PCWL119, and PCWL120, respectively from Arabidopsis stem cDNA (Supplementary table S1) and cloned into pDONR™ 207 using the Gateway BP Clonase II enzyme mix (11,789, Invitrogen, Canada). These expression clones were transformed into Agrobacterium GV3101 strain, colonies were confirmed by PCR and used for stable and transient expression in plant.

Subcellular localization

Agrobacterium containing 35S::GFP-AT1G28580 and plasma membrane marker CD3-1007 i.e. Plasma Membrane Intrinsic Protein 2a (PIP2a) (Nelson et al. 2007) was incubated at 28 °C, the culture was centrifuged at 6000 rpm, the pellet was resuspended in a mixture of 10 mM MES buffer (RM1128, HiMedia, India) and 10 mM MgCl₂ (MB237, HiMedia, India) pH -5.6 and diluted to 0.4 OD. 100 µM Aces- tosyringone was added to the suspension and kept overnight at room temperature. The leaves of Nicotiana benthamiana and Arabidopsis thaliana were then infiltrated with the sus- pension (Li 2011) and at 2nd or 3rd day infiltrated leaf sections were analyzed in LEICA SP8 confocal microscope. For plasmolysis experiment, infiltrated leaf sections were dipped in 30% glycerol for 30 min under vacuum.

Protein extraction and immunoblotting

The total proteins, soluble proteins, and wall or membrane-bound proteins extracted from Nicotiana leaf infiltrated with 35S::GFP-AT1G28580 and control leaves infiltrated only with infiltration buffer (MESK + MgCl₂). Leaf discs were crushed in 8 M urea for total protein extraction, centrifugated at a 16,000 rpm for 10 min, and the supernatant was collected as total protein fraction. For soluble protein fractions, leaf discs were first crushed in 50 mM sodium phosphate buffer (pH-6.8) containing 2.5 mM EDTA and 2% PVP. The supernatant was collected as a soluble protein fraction after centrifuging at high speed for 10 min. The residual pellet was then crushed in 8 M urea for extraction of membrane bound proteins. The total protein concentration of all protein fractions was determined by the Bradford assay. The equal amount of protein (25 µg) mixed with 4 x Laemmli buffer (0.1 M Tris, pH 6.8, 20% w/v glycerol, 4% w/v SDS, 100 mM DTT, and 0.001% w/v Bromophenol blue) and heated at 95 °C for 10 min. The protein preparations of all fractions were run on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane by wet transfer procedure. Then, membrane blocking was done using 5% (w/v) skimmed milk powder in 1 x TBST. The blots were developed and imaged under ImageQuant LAS 4000.

The total protein fractions were then washed three times with 1 x TBST and incubated with Goat Anti-Rabbit (BB-SAB01A, BioBharti LifeSciences Pvt. Ltd., India) primary antibody. And the lower half membrane with low molecular weight proteins was incubated with anti-GAPDH (PAB932Mu01, CloudClone Corp., USA) primary antibody. The next day, blot were washed three times with 1 x TBST and incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at RT. The blots were again washed with 1 x TBST and using Luminata™ Forte western HRP substrate (WBLUF0100, Merck Millipore, Germany), blots were developed and imaged under ImageQuant LAS 4000 (GE Healthcare). The blot that was probed with anti-GAPDH was washed three times with stripping buffer (NaCl, glacial acetic acid, and water), again washed with 1 x TBST, and incubated overnight at 4 °C with anti-V-ATPase (AS07213, Agrisera, Sweden) primary antibody and next day after wash- ing with 1 x TBST incubated with Goat Anti-Rabbit secondary antibody for 1 h at RT which was developed with HRP substrate and imaged under ImageQuant LAS 4000.
Genotyping of T-DNA mutant

SALK_030805 line of AT1G28580 was obtained from Arabidopsis Biological Resource Centre (ABRC), and homozygous lines were isolated by genotyping using LP(PCWL99), RP (PCWL100), and BP(PCWL29) primers (Supplementary table S1).

The total RNA extraction using trizol method and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Fresh tissue was collected from Arabidopsis rosette leaf and stem and ground in liquid nitrogen. RNA was extracted using Trizol method (15,596,018, Invitrogen, Canada) and extracted RNA was treated with TURBO DNA-free kit (AM1907, Invitrogen, Lithuania). cDNA synthesis of the DNAs treated 1 μg RNA samples was done using iScript cDNA Synthesis kit (1,708,891, BioRad, USA). Using ACTIN2 (AT3G18780) as reference gene, the expression of MYB46 (AT5G12870), PAL2 (AT3G53260), and AtGELPs from clade Id and Ic were measured using qPCR primers listed in supplementary table S1. The relative fold change was calculated by ΔΔCt method.

Generation of Arabidopsis transgenic lines

The Agrobacterium culture containing 35S::AT5G12870 (MYB46) and 35S:AT1G28580 (AtGELP7) plasmids were grown at 28 °C for 2 days, pelleted at 6000 rpm for 15 min and suspended in transformation medium consisting of 5% sucrose (GRM3063, HiMedia, India) and 0.05% silwet-77 (PCT1554, HiMedia, India) suspension. The floral dip transformation was performed as explained in (Clough and Bent 1998). When the plants were completely dried, seeds were collected and selected against glufosinate ammonium (BASTA) (C45520, Sigma-Aldrich, Switzerland). The homozygous lines from T3 generation were used for further experiments.

Staining and imaging of Arabidopsis stem sections

Wild-type and transgenic Arabidopsis stem sections were stained with Toluidine Blue O (T3260, Sigma-Aldrich, USA) and imaged under Nikon DS-Qi2 ECLIPSE Ti fluorescence microscope under 10X and 40X magnification (Pradhan Mitra and Loqué 2014).

Esterase activity

With p-nitrophenyl acetate

The soluble and wall bound proteins were extracted from stem and leaf tissue as explained by (Biswal et al. 2014). The total protein concentration was determined by Bradford assay. Esterase activity was performed by p-nitrophenyl acetate (18432, SRL, India) as substrate, incubated at 37 °C for 2 h, released 4-nitrophenol quantified at 400 nm and specific esterase activity was calculated in nmol per min per mg of total protein using 4-nitrophenol standard curve.

With acetylated xylan as a substrate

The soluble and wall-bound proteins were extracted from stem tissue as explained by (Biswal et al. 2014). The total protein concentration was determined by the Bradford assay. Esterase activity was performed with partially acetylated xylan (P-ACXYL, Megazyme, Ireland) as substrate, incubated at 37 °C for 6 h, released acetic acid quantified by acetic acid kit (K-ACET, Megazyme, Ireland).

Lipase activity

The soluble and wall bound proteins were isolated from Nicotiana leaf tissue as described by (Gupta et al. 2002). Lipase activity was performed with 4-Nitrophenyl palmitate (N2752, Sigma-Aldrich, Mongolia) as substrate, incubated at 50 °C for 5 h, and released 4-nitrophenol quantified at 410 nm. Then using the BSA standard curve, the specific lipase activity was calculated in nmol of product releases per min per mg of total protein using 4-nitrophenol standard curve.

Preparation of alcohol insoluble residue (AIR)

The dried plant tissue was ground in Qiagen TissueLyser II, treated with 80% ethanol in 4 mM HEPES buffer (MB016, HiMedia, India) for 30 min at 70 °C. The mixture was then centrifuged and pellet was sequentially washed with 800 ul of 70% ethanol, chloroform: methanol (1:1) and acetone. After removing acetone, the pellet was dried overnight.
Cell wall acetyl content analysis

1 mg of AIR sample was saponified using 1 M NaOH, and neutralized with 1 M HCl, and saponified supernatant was analyzed by acetic acid kit (K-ACET, Megazyme, Ireland).

Sequential extraction of pectin, xyloglucan, and xylan

Leaf AIR was incubated with 50 mM ammonium formate (50504, SRL, India) buffer at 37 °C for 24 h in shaker dry bath. The supernatant ammonium formate fraction was collected, and the pellet was digested with pectate lyase (E-PCLYAN2, Megazyme, Ireland). Both ammonium formate and pectate lyase fractions were freeze-dried, and the pellet was dissolved in water. After pectin extraction, the pellet containing xyloglucan and xylan was digested with endoglucanase i.e., cellulase (C1184-5KU, Sigma-Aldrich, USA) to remove xyloglucan and the remaining pellet rich in xylan was freeze dried. Finally, pectin fraction, combined xyloglucan and xylan-rich fraction, and xylan-rich fraction were analyzed for acetic acid (K-ACET, Megazyme, Ireland), galacturonic acid (K-URONIC, Megazyme, Ireland), and xylose (K-XYLOSE, Megazyme, Ireland) content.

DMSO extraction of xylan

AIR samples were delignified using 1.3% sodium hypochlorite (Q27905, Thermo Fisher Scientific, India), incubated at 75 °C for 2 h with shaking in dry bath. Samples were centrifuged, pellet was washed with acetone and dried overnight. DMSO was added to above holocellulosic fraction and incubated at 60 °C for 24 h in shaking dry bath. Supernatant collected after centrifugation was precipitated in ethanol:methanol:water (7:2:1) mixture by keeping at 4 °C for 2 days. Precipitated xylan was centrifuged, pellet was washed with acetone and dried overnight. This DMSO extracted xylan was used for further analysis.

XOS analysis by MALDI-TOF–MS

3 mg of AIR from dried stem tissue was digested with GH11 endo-1,4-β-Xylanase (E-XYLAA, Megazyme, Ireland) at 60 °C for 2 days and the remaining hydrolysate was purified as explained in (Chong et al. 2011). Both neutral and acidic XOS fractions were subjected to MALDI analysis separately.

Cell wall composition

Xylose content: 2 mg of AIR sample was treated with 100 µl of 1.3 M HCL, incubated in dry bath at 100 °C for 1 h. The samples were neutralized with 100 µl of 1.3 M NaOH and final volume was made up to 1 ml by adding MilliQ water, centrifuged at 1500 g for 10 min. 50 µl supernatant was used for xylose analysis and quantified by Megazyme Kit (K-XYLOSE, Megazyme, Ireland).

Cellulose content: AIR sample was digested with α-amylase (A3176, Sigma-Aldrich, USA) at 45 °C to remove starch. Destarched cell wall residue was used to generate glucose monomer which was quantified by anthrone assay (Updegraff 1969).

Acetyl Bromide Soluble Lignin (ABSL): AIR samples were incubated with freshly prepared 25% acetyl bromide (135,968, Sigma-Aldrich, Mongolia) containing acetic acid at 50 °C for 2 h. The supernatant was diluted with 2 M NaOH and freshly prepared hydroxylamine hydrochloride (159,417, Sigma-Aldrich,). The absorbance was taken at 280 nm and lignin content was represented in mg per g of AIR (Foster et al. 2010).

Saccharification analysis

Alkali pre-treatment: AIR sample was treated with 0.4 M NaOH at 90 °C for 30 min. The supernatant was removed by centrifugation, the pellet was washed with water and acetone and dried for further analysis.

Xylanase pre-treatment: AIR from dried stem tissue was digested with GH11 endo-1,4-β-Xylanase (E-XYLAA, Megazyme, Ireland) at 60 °C for 48 h. The digested pellet was washed with acetone, dried and used for further analysis.

Preparation of enzyme mix for saccharification: 40 U/ml of each cellulase (C1184-5KU, Sigma-Aldrich, USA) and β-glucosidase (E-BGLUC, Megazyme, Ireland) were prepared in 0.1 M acetic acid buffer (pH-4.8) with 1:2 separately. The desalted individual enzyme buffer mixture (PD-10 17085, GE Healthcare, UK) was mixed in 8:2. Finally, the enzyme mix was diluted ten times with 0.1 M acetic acid buffer (pH-4.8) and used for saccharification.

Enzymatic hydrolysis: The untreated or treated cell wall powder was suspended in enzyme mix as explained earlier. All the samples were incubated at 50 °C for 96 h, centrifuged, the glucose in supernatant was analyzed by Glucose.
oxidase (GOD)- Peroxidase (POD) assay (Acker et al. 2016).
To analyze the glucose content, the sample collected after
saccharification was incubated with GOD-POD solution at
37 °C for 30 min. This GOD-POD mixture was prepared by
mixing glucose oxidase (61,788, SRL, India), peroxidase ex. Horseradish (73,292, SRL, India), 2,2'-Azino-bis (3-ethyl-
benzothiazoline-6-sulfonic acid) (A9941, Sigma-Aldrich, USA) in 0.1 M acetic acid buffer (pH 4.5). The absorbance
was measured after incubation at 405 nm. Glucose content
was calculated using standard curve.

Statistical analysis

The p value was calculated using Student’s t-test in Office Excel 365.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s11103-022-01275-8.

Acknowledgements

The plasmid sequencing and MALDI was performed at Advanced Technology Platform Centre (ATPC), Faridabad.
pCC0995 vector was provided by Dr Clint Chapple (Purdue University, USA) pDONRTM207, pSITE-2CA, CD3-1007 vectors and western blot antibodies were provided by Dr. Saikat Bhattacharjee (Regional Centre for Biotechnology, Faridabad). Prince Singh, Mansi Srivastav and Bhagwat assisted us in few experiments. Suraj Tiwari provided technical assistance during confocal imaging. Jaydeep Sharma and Krishna Singh Bisht for technical assistance in western blotting.

Author contributions

PM-AP designed and conceptualized the research, LR performed most of the experimental analysis. AAC performed saccharification assay. RS performed initial cloning experiment. PM-AP and LR wrote the manuscript. All authors have read and agreed to publish the manuscript.

Funding

This work is supported by DST-INSPIRE faculty fellowship (DST/INSPIRE/04/2018/000215) and SERB-SRG Grant (SRG/2020/000861).

Data availability

Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest

Authors declare no conflict of interest.

Consent to participate

All authors agree.

Consent for publications

All authors agree.

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