Arabidopsis GELP7 is plasma membrane-localized acetyl xylan esterase, and its overexpression improves saccharification efficiency.

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Research Article

Keywords: Acetyl xylan esterase, plant cell wall, deacetylation, saccharification, GELP7, Arabidopsis, GDSE esterase

Posted Date: September 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-847876/v1>

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Version of Record: A version of this preprint was published at Plant Molecular Biology on May 17th, 2022. See the published version at <https://doi.org/10.1007/s11103-022-01275-8>.

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 a decrease in xylan acetylation in comparison with wild type plants. Therefore, we have named this enzyme as *AtAXE1*. The subcellular localization 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*, which is 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.

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 lead to 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 of the 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 BRITTLE LEAF SHEATH 1 (BS1) and DEACETYLASE ON ARABINOSYL SIDECHAIN OF XYLAN 1 (DARX1). BS1 specifically deacetylates xylan, which is a major hemicellulose found in the secondary cell walls (Zhang et al. 2017) whereas DARX1 specifically cleaves acetyl groups present on arabinosyl residues of xylan backbone (Zhang et al. 2019). Both of these enzymes are localized in Golgi and involved in regulating the pattern of xylan acetylation. Other proteins which belong to the reduced wall acetylation (RWA) family transports acetyl-CoA or any other acetyl carrier from cytoplasm to Golgi (Pauly and Ramírez 2018). Golgi localized ALTERED XYLOGLUCAN 9 (AXY9) and TRICHOME BIREFRINGENCE-LIKE (TBL) family belong to the polysaccharide acetylation pathway. Arabidopsis *axy9* mutant showed decreased xylan and xyloglucan acetylation (Schultink et al. 2015). TBL is a multigene family and has acetyltransferase activity to specific polysaccharides. Arabidopsis *tbl29*, *tbl32* and *tbl33* mutants have decreased levels of xylan acetylation (Xiong et al. 2013; Yuan et al. 2016). Golgi synthesized acetylated xylan is transported to apoplastic space and probably deacetylated by apoplastic localized AXEs. There are many uncharacterized GELPs in plants that might have a role in cell wall polysaccharide deacetylation. In Arabidopsis, no member from the GELP family having a role in cell wall polysaccharide deacetylation have been reported so far. GELP family members have a flexible enzyme active site that gives specificity to many substrates, hence contributing multiple cellular functions in plant. GELP amino acid sequence consists of five (I-V) consensus sequences and four conserved amino acid residues, i.e., Ser(S)-Gly(G)-Asn (N)-His(H) found in blocks I, II, III, and V, respectively, which are involved in catalysis. Thus, GELP is categorized in the SGNH hydrolase superfamily (Akoh et al. 2004). *AtGELP33/AtFXG1* showed fucosidase activity, suggesting the GELP family role in cell wall modification (De la Torre et al. 2002). *AtGELP83/AtCDEF1* and *AtGELP60/AtLTL1* are involved in cutin biosynthesis and de-esterification respectively (Naranjo et al. 2006; Takahashi et al. 2010). *AtGELP63/AtESM1* is involved in glucosinolate hydrolysis (Zhang et al. 2006). Rice GELPs i.e., BS1 and DARX1 are AXEs as mutation in these genes led to an increase in xylan acetylation. Rice GELP members i.e., MAO HUI 11 (MHZ11) and 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, *AtGELP7* or *At1G28580* 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, sequences from clade Id showed high sequence identity from 28–42% with rice BS1 (Fig.S3). All the members from clade Id had either TAP-like or acetylhydrolase domain. This suggested that this clade could be involved in xylan deacetylation.

At GELP7 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 – 2 (OE) 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, which was correlating with previous findings (Fig. 1C) (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 by around 55-fold in both MYB46 OE lines (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*. *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 where *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 similar lipase activity was observed in empty vector control, *AtAXE1* and *AnAXE1* expressed *Nicotiana* leaves (Fig. 2B). This signifies that *AtAXE1* is an esterase.

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 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. Bioinformatic analysis showed *At1G28580* or *AtAXE1* has a signal peptide and two transmembrane domains. (Fig.S4 and Fig.S5). Altogether this data demonstrated 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). The T-DNA insertion was found at the 3' region of *AtAXE1* gene, suggesting that this could be a knockdown mutant (Fig. 3A). Homozygous mutants were isolated by genotyping, which morphologically look similar to the wild type plant (Fig. 3B and Fig. 3C). 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 plant respectively (Fig. 3D and Fig. 3E). 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, but no significant difference in stem tissue was observed as compared to the wild-type plant (Fig.S6B and Fig.S6C). Based on bioinformatics analysis, our hypothesis was *AtAXE1* could be a polysaccharide esterase. Therefore, total cell wall acetyl content was measured in leaf and stem. The acetyl content was unchanged in the leaf but increased in the stem by 12% in *ataxe1* Arabidopsis mutant (Fig. 3F and Fig. 3G). 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.S6D and Fig.S6E). The wavenumber 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 an 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 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.S7). Overexpression of the *AtAXE1* gene was observed in leaf and stem tissue of transgenic lines by RT-PCR where 5b line showed the highest expression in both tissues in comparison with lines 2b and 3b (Fig. 4C and Fig. 4D). 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 Fig. 4F).

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* could be involved in polysaccharide deacetylation.

Sequential extraction and FT-IR 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. 5D). To confirm these results, stem powder samples were subjected to FTIR analysis. Spectral analysis showed less abundant $1210\text{--}1275\text{ cm}^{-1}$ (C-O-C) acetyl linkage in transgenic lines in comparison with wild type of both leaf and stem (Fig. 5E and Fig. 5F). The spectral range $1730\text{--}1750\text{ cm}^{-1}$ corresponding C = O acetyl linkage was also reduced in stem tissues of transgenic plants (Fig. 5f). We further calculated the ratio of acetyl to xylose linkage, and it was decreased in transgenic lines as compared with wild type (Fig. 5G and Fig. 5H). In conclusion, sequential extraction and FT-IR data confirmed that *AtAXE1* is acetyl xylan esterase.

Xylanase digested *AtAXE1* transgenic lines revealed an increase in xylan digestibility and confirms 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). Acidic XOS ranging from DP3 to DP5 are acetylated and substituted with either glucuronic acid or methyl glucuronic acid (Fig. 6B). 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 xylo-tetraose 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. 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* is 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 5b line than in WT (Fig. 7A). In another experiment, the cell wall powder was pretreated with 0.4M 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 recalcitrance. 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.

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 member family, and a few members of this family are characterized which play a role in ethylene signaling, 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 methylesterase, 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 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 weaker cell wall. Extracellular AXEs can maintain acetylation level 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 (Fig. 2C). The *AtAXE1* was further characterized by generating transgenic lines overexpressing *AtAXE1*. The esterase activity was increased in transgenic lines and acetyl content was reduced in both leaf and stem tissue (Fig. 4). 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. 5). This confirmed that *AtAXE1* is acetyl xylan esterase. 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. 5). In conclusion, these data confirmed that *AtGELP7* is acetyl xylan esterase.

Additionally, we observed compensatory increase in pectin acetylation in highly expressed line 5b (Fig. 5A). Altering polysaccharide xylan acetylation often led to increase in pectin acetylation and substitution of glucuronic acid on xylan chain (Xiong et al. 2015). 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.S7). 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 *ataxe1* mutant also showed increase in stem acetylation by 12% because of less AXE activity (Fig. 3). Increase in stem acetylation led to dwarfism in rice BS1 mutants because of vessel cell spatial patterning but this was not observed in *ataxe1* 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). Upregulation 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 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). 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 project, *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 Fig. 7B). 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* which is 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 pDONRTM 207 using the Gateway BP Clonase II enzyme mix (11789, Invitrogen, Canada). This pENTR-*AT1G28580* and pENTR-*AT5G12870* were further subcloned into destination vectors pCC0995 containing constitutive 35S promoter (*35S::AT1G28580*, *35S::AT5G12870*) (Weng et al. 2010) and pSITE-2CA (*35S::GFP-AT1G28580*) (Chakrabarty et al. 2007) containing N-terminal tagged GFP vector using the Gateway LR Clonase II enzyme mix (11791, 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* (Nelson et al. 2007) was incubated at 28°C, the culture was centrifuged at 6000 rpm, the pellet was resuspended in a mixture of 10mM MES buffer (RM1128, HiMedia, India) and 10mM MgCl₂ (MB237, HiMedia, India) pH-5.6 and diluted to 0.4 OD. 100 mM Acetosyringone was added to the suspension and kept overnight at room temperature. The leaves of *Nicotiana benthamiana* were then infiltrated with the suspension (Betsuyaku and Sawa 2009) 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.

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 (15596018, Invitrogen, Canada) and extracted RNA was treated with TURBO DNA-free kit (AM1907, Invitrogen, Lithuania). cDNA synthesis of the DNase treated 1 mg RNA samples was done using iScript cDNA Synthesis kit (1708891, 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 $\Delta\Delta C_t$ 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

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.

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 mins 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 1M NaOH, and neutralized with 1M 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 50mM ammonium formate (50504, SRL, India) buffer at 37°C for 24 h in shaker dry bath. The supernatant ammonium formate fraction was collected, and 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. Then 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.

Fourier Transform Infrared (FTIR) spectroscopy analysis

Qiagen TissueLyser II milled dried stem powder and AIR from fresh leaf tissues were placed on ZnSe ATR crystal in Bruker Tensor27 and scanned 16 times to get the absorbance peaks of different functional groups at wavenumbers ranging from 800 cm^{-1} to 2000 cm^{-1} . Acetyl linkages were detected at 1240 cm^{-1} , and 1740 cm^{-1} and xylose linkages were detected at 976 cm^{-1} , 1037 cm^{-1} , and 1073 cm^{-1} by analyzing birchwood xylan (partially acetylated) (P-ACXYL, Megazyme, Ireland) and beechwood xylan (P-XYLNBE, Megazyme, Ireland) as controls.

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 1.3M HCL, incubated in dry bath at 100°C for 1 hr. The samples were neutralized with 100 μl of 1.3M NaOH and final volume was made up to 1ml by adding MilliQ water, centrifuged at 1500g for 10 mins. 50 μl supernatant was used for xylose analysis and analyzed 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 analyzed by anthrone assay (Updegraff 1969).

Acetyl Bromide Soluble Lignin (ABSL): AIR samples were incubated with freshly prepared 25% acetyl bromide (135968, Sigma-Aldrich, Mongolia) containing acetic acid at 50°C for 2 h. The supernatant was diluted with 2M NaOH and freshly prepared hydroxylamine hydrochloride (159417, 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 hrs. The pellet was washed with acetone, dried and used for further analysis.

Preparation of enzyme mix for saccharification: Equimolar enzyme solutions (40U/ml) were prepared for both cellulase (C1184-5KU, Sigma-Aldrich, USA) and β -glucosidase (E-BGLUC, Megazyme, Ireland). The enzyme solutions were then mixed with 0.1 M acetic acid buffer (pH- 4.8) in ratio 1:2 separately. The individual solution desalted through PD 10 column (17085101, GE Healthcare, UK). The desalted cellulase and β -glucosidase was then mixed in 8:2 ratio to prepare enzyme mix. Finally, the enzyme mix was diluted ten times with 0.1 M acetic acid buffer (pH- 4.8) to make a diluted enzyme mix for saccharification.

Enzymatic hydrolysis: The untreated or treated cell wall powder was suspended in reaction mixture containing 0.1 M acetic acid buffer (pH- 4.8) and enzyme mix (8:2) of cellulase and β - glucosidase. 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 mins. This GOD-POD mixture was prepared by mixing glucose oxidase (61788, SRL, India), Peroxidase ex. Horseradish (73292, SRL, India), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (A9941, Sigma-Aldrich, USA) in 0.1M 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.

Declarations

Acknowledgments

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 and pSITE-2CA vectors were provided by Dr. Saikat Bhattacharjee (Regional Centre for Biotechnology, Faridabad). Prince Singh and Mansi Srivastav assisted us in few experiments. Suraj Tiwari provided technical assistance during confocal imaging.

Funding

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

Author contributions

PM-AP designed 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.

Conflicts of interest

Authors declare no conflict of interest

Consent to participate

All authors agree

Consent for publications

All authors agree

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Figures

Figure 1

Representation of MYB46 overexpressor line and expression of Arabidopsis GELPs in 35S::MYB46 overexpressing 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 overexpressing MYB46 transgenic lines, MYB46 OE-2 and MYB46 OE-3. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at $**p \leq 0.05$, $*p \leq 0.1$.

Figure 2

Transient 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. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at **p $\leq$ 0.05, * p $\leq$ 0.1.

Figure 3

Screening of T-DNA mutant line of *AtAXE1*, referred as *ataxe1*. A Gene structure of *AtAXE1* showing SALK_030805 T-DNA insertion at 3' UTR. B Agarose gel showing homozygous line confirmed by LP, RP and BP primers. C Picture of 6 weeks old Arabidopsis wild type and *ataxe1* mutant. RNA level expression of *AtAXE1* (D) in leaf, and (E) in stem tissue of homozygous mutant plant by RT-PCR. Wild-type and mutant Arabidopsis (F) leaf, and (G) stem alcohol insoluble residues saponified and acetyl content was

analysed by acetic acid kit (K-ACET) from Megazyme. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at $**p \leq 0.05$, $*p \leq 0.1$.

Figure 4

Evaluation of three independent transgenic lines of Arabidopsis overexpressing 35S::AtAXE1. A Expression data of AtAXE1 gene in Arabidopsis plant from eFP browser (<http://bar.utoronto.ca/>). B Picture of wild-type and three independent transgenic lines. Expression of AtAXE1 gene in (C) leaf tissue,

and (D) stem tissue of transgenic lines by RT-PCR. Esterase activity in extracted soluble and wall bound protein fractions incubated with 4-nitrophenyl acetate and released product 4-nitrophenol was analysed by spectrophotometer at 400 nm (E) in leaf tissue, and (F) in stem tissue. Wild-type and transgenic Arabidopsis (G) leaf, and (H) stem alcohol insoluble residues saponified and acetyl content was analysed by acetic acid kit (K-ACET) from Megazyme. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at $**p \leq 0.05$, $*p \leq 0.1$.

Figure 5

Sequential extraction of different cell wall polymers and acetyl content analysis in (A) pectin fraction, (B) combined xyloglucan and xylan-rich fraction, (C) xylan-rich fraction, and (D) DMSO extracted xylan fraction. Fourier transform infrared (FTIR) spectroscopy was performed in (E) leaf, and (F) stem tissue of wild type and transgenic Arabidopsis plant, to detect different bonds in cell wall material. The red arrow represents the significant decrease in acetyl-ester linkages. The ratio of wavenumbers corresponding acetyl-ester to xylose peaks in (G) leaf, and (H) stem. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at $**p \leq 0.05$, $*p \leq 0.1$.

Figure 6

Xylan oligosaccharide analysis in (A) neutral XOS, (B) acidic XOS, released by GH11 β -endo-xylanase and analyzed by MALDI. Arrows indicate trend of XOS release. Showing this data from one of experiment which is repeated twice.

Figure 7

Improved saccharification of stem lignocellulose in overexpressing 35S::AtAXE1 Arabidopsis transgenic lines. Graphs showing glucose release after enzymatic hydrolysis (A) without pre-treatment, (B) after NaOH pre-treatment, and (C) with pre-treatment by xylanase. Analysis of major cell wall components in WT and transgenic line stem powder (D) cellulose, (E) xylose (Megazyme D-Xylose Kit), and (F) lignin. Data represents mean $\pm$ SE, n = 3-4 biological replicates, Student's t-test at $**p \leq 0.05$, $*p \leq 0.1$

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