

Structure-Activity Relation of AMOR Sugar Molecule That Activates Pollen-Tubes for Ovular Guidance¹[OPEN]

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Successful fertilization in flowering plants depends on the precise directional growth control of pollen tube through the female pistil tissue toward the female gametophyte contained in the ovule for delivery of nonmotile sperm cells. Cys-rich peptides LUREs secreted from the synergid cells on either side of the egg cell act as ovular attractants of pollen tubes. Competency control by the pistil is crucial for the response of pollen tubes to these ovular attractants. We recently reported that ovular 4-*O*-methyl-glucuronosyl arabinogalactan (AMOR) induces competency of the pollen tube to respond to ovular attractant LURE peptides in *Torenia fournieri*. The beta isomer of the terminal disaccharide 4-*O*-methyl-glucuronosyl galactose was essential and sufficient for the competency induction. However, critical and noncritical structures in the disaccharide have not been dissected deeply. Herein, we report the synthesis of new AMOR analogs and the structure-activity relationships for AMOR activity in the presence of these synthesized analogs. Removal of 4-*O*-methyl group or -COOH from the glucuronosyl residue of the disaccharide dramatically reduces AMOR activity. The pyranose backbone of the second sugar of disaccharide is essential for the activity but not hydroxy groups. The role of beta isomer of the disaccharide 4-Me-GlcA- β (1,6)-Gal is very specific for competency control, as there was no difference in effect among the sugar analogs tested for pollen germination. This study represents the first structure-activity relationship study, to our knowledge, of a sugar molecule involved in plant reproduction, which opens a way for modification of the molecule without loss of activity.

Fertilization in flowering plants is a complex process that involves several steps, starting from the landing of the pollen on the female stigma to the formation of a pollen tube, which is then precisely guided by the pistil tissue for the delivery of nonmotile sperm cells to the female gametes (Fig. 1A). Research spanning a decade has revealed that the pollen tubes communicate extensively with the female sporophytic tissue that provides various guidance cues to enable them to find their way to the ovules (Higashiyama and Takeuchi, 2015). The

ovules also secrete Cys-rich peptides named LUREs that attract the pollen tubes to them before fertilization (Okuda et al., 2009). The concept of competency control of pollen tubes was established with the findings that in vitro-germinated pollen tubes of *Torenia* (*Torenia fournieri*) are not capable of being guided toward the ovules placed in front of them or to the gelatin beads releasing the LURE attractant peptides (Higashiyama et al., 1998; Okuda et al., 2013). However, the pollen tubes growing through cut, pollinated style of sufficient length responded to the LURE peptides in a semi-in vitro assay, suggesting that unknown factors from the female sporophytic tissues provides competency to the pollen tubes and primes them to respond to the attraction signal (Okuda et al., 2013). In *Arabidopsis* (*Arabidopsis thaliana*), an established plant model to study ovular pollen tube guidance (Higashiyama and Takeuchi, 2015), pollen tubes gain higher competency upon contact with the stigma and style (Palanivelu and Preuss, 2006). Though the requirement of female tissues for competency control was established in these different plant species, the female molecule responsible for competency control remained elusive for a decade.

In our efforts to identify the female molecule responsible for competency control, we discovered an ovular arabinogalactan polysaccharide, named 4-*O*-methyl-glucuronosyl arabinogalactan (AMOR),

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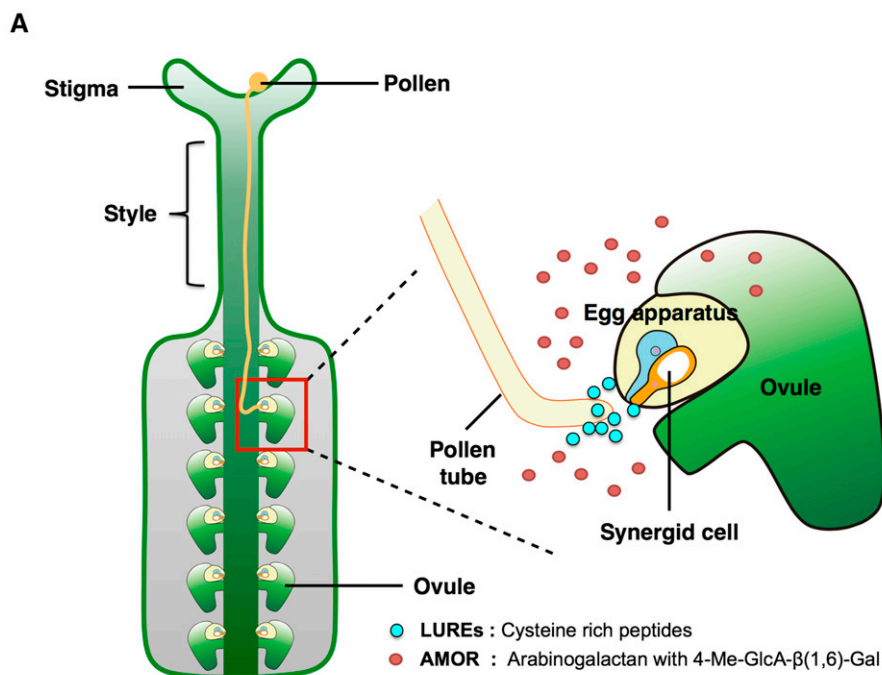
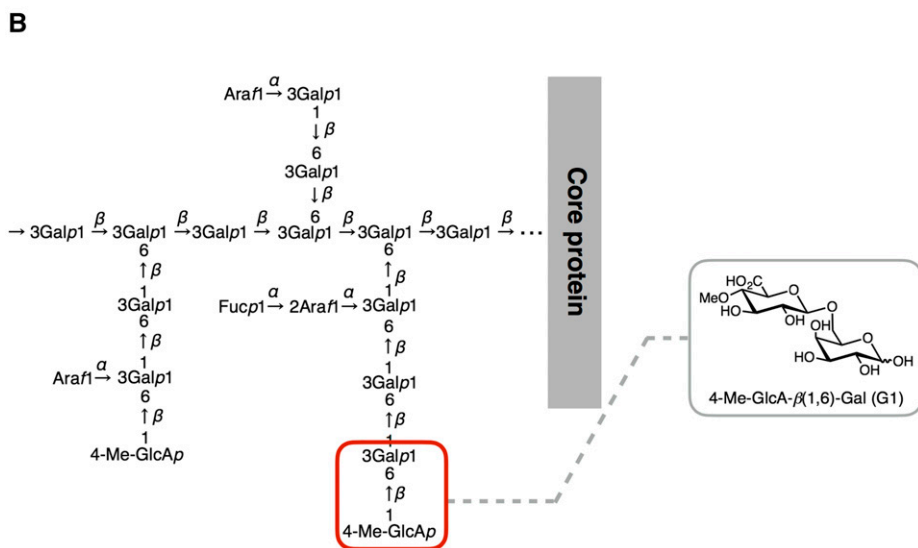


Figure 1. Ovular factor AMOR induces competency in pollen tubes to respond to ovular attractant. A, Schematic of pollen tube growing through a *Torenia* pistil toward an ovule. The pollen tube gain competency in presence of the ovular factor AMOR arabinogalactan that enables it to respond to the LURE attractant peptides secreted from the synergid cell. B, The terminal residue (4-*O*-methyl-glucuronosyl residue, highlighted by a red square) of an arabinogalactan polysaccharide shows AMOR activity.



which induces competency of the pollen tubes to respond to ovular attractant LURE peptides in *Torenia* (Mizukami et al., 2016; Fig. 1A). Competency control in *Torenia* is two-step; pollen tubes have to grow through the style tissue (first step) before they can gain full competency by AMOR in the ovary (second step). The AMOR activity was found to reside in the terminal 4-*O*-methyl-glucuronosyl residue of the arabinogalactan polysaccharide. A chemically synthesized disaccharide, the beta isomer of 4-*O*-methyl-glucuronosyl Gal (4-Me-GlcA-β(1,6)-Gal (G1; Fig. 1B) showed AMOR activity and conferred competency to respond to ovular attractant LUREs (Mizukami et al., 2016).

Arabinogalactan polysaccharides are found to be associated with arabinogalactan proteins (AGPs), a complex family of glycoproteins, known as Hyp-rich

glycoproteins, that play a major role in various aspects of plant growth, development, and reproduction (Seifert and Roberts, 2007; Ellis et al., 2010; Geshi et al., 2013; Pereira et al., 2014). AGPs are very heterogeneous, with the glycan constituting up to 90% of the mass of the molecule. The glycan part consists of variable side chains of Gal and arabinose sugars that are linked to O6 residue of the β-1,3-linked galactan backbone (Geshi et al., 2013). The galactan backbone is in turn added on to the proteins using *O*-glycosylation onto Hyp residues in the AGP backbone (Ogawa-Ohnishi and Matsubayashi, 2015). Most of the AGPs are found to have a GPI anchor at the C terminus that allows it to be anchored to the membrane and function as signaling molecules (Borner et al., 2002; Schultz et al., 2004). It has

been proposed that AGPs are released to the extracellular matrix by the cleavage of GPI anchor by specific phospholipases (C and D), allowing it to act as a signal by itself or to activate downstream signal transduction pathways by interacting with other proteins (Gaspar et al., 2001; Schultz et al., 2004). There also exists a possibility of carbohydrate moieties being released from the AGPs via cleavage by specific enzymes, which allows these saccharides to function as signaling molecules that activate various signal transduction pathways by binding to specific receptors (Showalter, 2001). A chemical biology approach could enable us to better understand the developmental signaling pathways that are activated by the saccharide moieties of the AGPs.

In this study, we report the structure-activity relationship (SAR) studies of the AMOR disaccharide molecule (**G1**) that activates pollen tubes for ovular guidance in *Torenia*. Using a method for assaying response capability of a pollen tube by micro-manipulating an ovule of *Torenia*, we analyzed the activity of various synthetic analogs of **G1** that induces competency of pollen tubes and revealed various residues that are critical and noncritical for AMOR activity. We also carried out a pollen tube germination assay with the close analogs of **G1** to assess if the AMOR activity was specific for competency control.

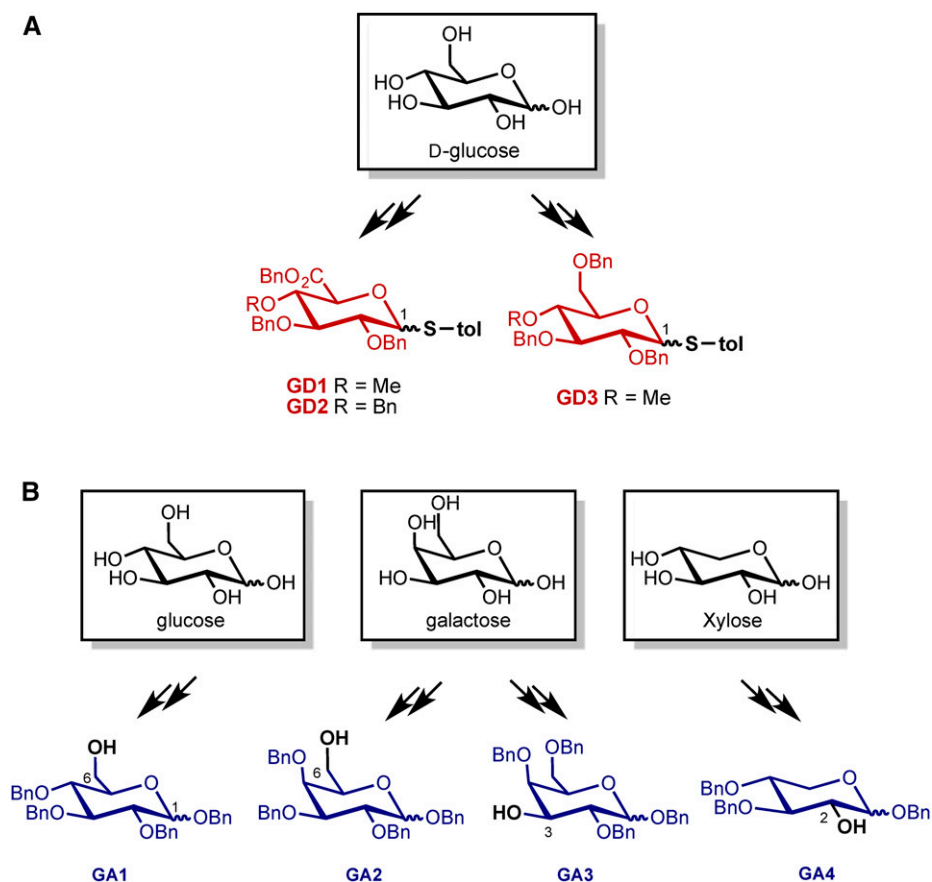
This work represents the first SAR study, to our knowledge, of a bioactive sugar molecule involved in plant reproduction.

RESULTS

Synthesis of **G1** and Its Analogs

The AMOR disaccharide **G1** found in the terminal exposed portion of arabinogalactan sugar chain consists of two sugar units, 4-*O*-methyl GlcA and Gal, linked together by β -1,6 linkage. In the previous work, we designed a methyl etherified (C-4 position) benzyl glucosyluronate trichloroacetimidate as the glycosyl donor and benzyl protected Gal with unsubstituted hydroxy group at C-6 position as the glycosyl acceptor. With these two glycosylation partners (glycosyl donor and acceptor), we successfully obtained the 1,6-glycoside with a good β -selectivity (Mizukami et al., 2016). Both donor and acceptor can be synthesized from readily available Glc and Gal via classical carbohydrate synthesis. To illustrate the structural features of the AMOR disaccharide for its activity, we systematically synthesized a series of analogs of **G1** and conducted SAR studies toward uncovering the key structural information (glycosidic bond and functional group on each sugar unit) that is essential for the AMOR activity.

Figure 2. Synthesis of glycosyl donors from Glc and glycosyl acceptors from monosaccharides. A, The synthesis of glycosyl donors from Glc was demonstrated. Three thioglycoside analogs **GD1**, **GD2**, and **GD3** as glycosyl donors were obtained by multistep synthesis. B, The glycosyl acceptors **GA1**, **GA2**, **GA3**, and **GA4** were synthesized from several commercial available saccharides.



Synthesis of Glycosyl Donors and Acceptors

For the programmable synthesis of AMOR disaccharide analogs, we first changed the glycosyl donors from imidate to thioglycosides (Zhang et al., 1999). Thioglycosides are more general and stable glycosyl donors showing broad substrate scope in glycosylation (Codée et al., 2005). Starting from Glc, we synthesized three glycosyl donors, **GD1**, **GD2**, and **GD3**, aiming to disclose the group effect of 4-*O*-methyl and carboxyl acid (COOH) moieties in glucuronosyl residue (Fig. 2A).

Four glycosyl acceptors (**GA1–GA4**) were synthesized from three widely commercial available monosaccharides (Fig. 2B). Each synthesized acceptor has one unprotected hydroxy group at a different position that was used for glycosylation with donors. This strategy allowed us to synthesize analogs that would reveal critical information about the second sugar unit (such as linkage effect) essential for AMOR activity.

Glycosylation and Deprotection of Disaccharide Analogs

Having a reasonably diverse set of glycosyl donors (**GD1–GD3**) and acceptors (**GA1–GA4**) in hand, they were cross-coupled in a combinatorial manner under the influence of NIS/TfOH (Vermeer et al., 2001) to generate protected disaccharides (Fig. 3). The desired beta anomers were isolated from major alpha glycosylation products, and the follow-up deprotection of benzyl groups on hydroxy groups by Pd/C-catalyzed hydrogenation (Oguri et al., 1995) afforded disaccharides **G1** to **G8**. A mild methylation of COOH group in **G1** by trimethylsilyldiazomethane (TMSCHN₂) gave its ester derivative 4,6-Me-GlcA-β(1,6)-Gal (**G9**; Presser and Hufner, 2004). To investigate the effect of hydroxy groups on a second sugar moiety (shown in blue), 4H-pyran-2-methanol was reacted with **GD1** to synthesize **G10**. Although the synthesis of a simple methyl glycoside 4-Me-GlcA-β-OMe (**G11**) was not straightforward (the reaction of **GD1** and methanol under NIS/TfOH

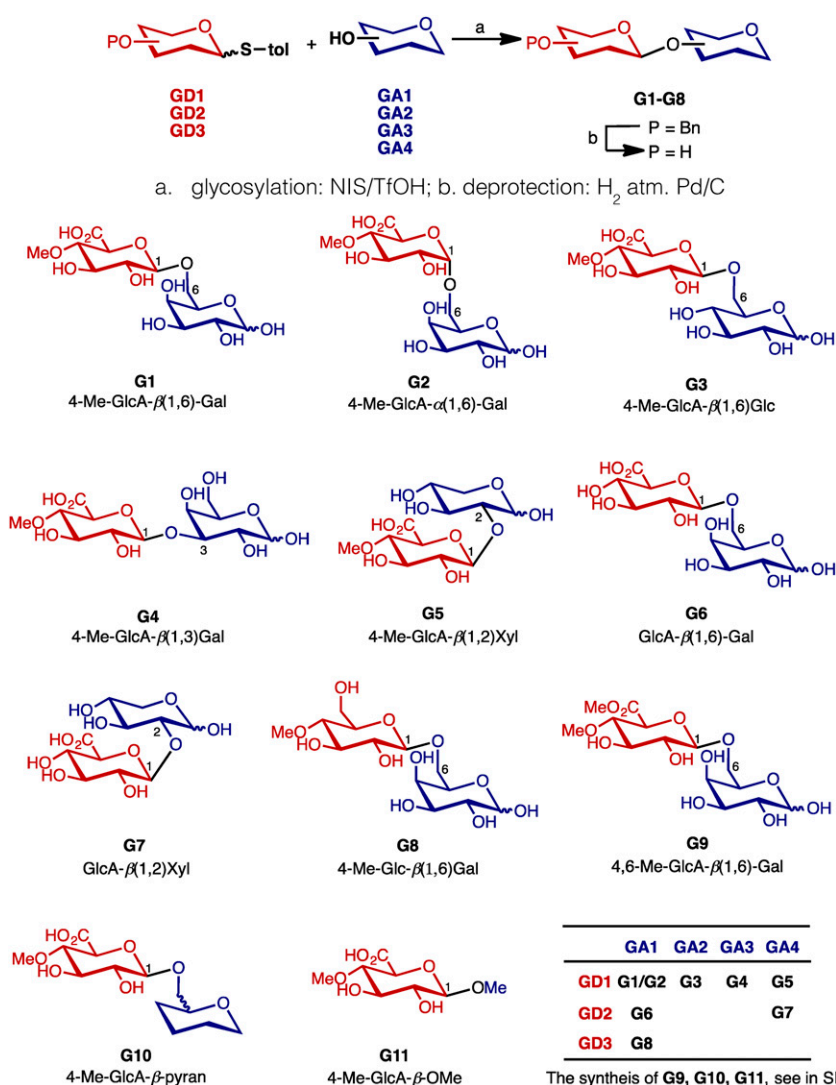


Figure 3. Glycosylation of donor and acceptor for disaccharides analogs. The program glycosylation is shown in the above scheme, using the synthesized glycosyl donor and acceptor mentioned in Figure 2. The structures of all disaccharides obtained are shown below; for **G1** to **G8**, the combinations of their original donor and acceptor were listed in the small table.

yielded inseparable mixtures after glycosylation), **G11** was synthesized from methyl β -D-glucopyranoside via a modified pathway (see Supplementary Information for details).

The characteristics of synthesized disaccharide analogs 4-Me-GlcA- α (1,6)-Gal (**G2**) to **G11** (Fig. 3) are as follows. **G2**, which has an α -glycosidic bond, is an anomer of **G1** (stereochemical importance of glycosidic bond). **G3** to 4-Me-GlcA- β (1,2)-Xyl (**G5**) have same 4-O-methyl glucuronosyl residue as **G1** but a different second sugar unit (**G1**: Gal, **G3**: Glc, **G5**: Xyl) or different linkage (**G1**: 1,6-linkage, **G4**: 1,3-linkage). For disaccharides β -glucuronosyl galactose (**G6**) and **G7**, the 4-O-methyl group on the glucuronosyl residue is lacking. Both **G8** and **G9** have modified COOH functionalities such as hydroxymethyl group (**G8**) and methyl ester group (**G9**; the effect of carboxylic acid moiety). The last two specific saccharides (**G10** and **G11**) with simple alkyl aglycones (**G10**: alkyl = $-\text{CH}_2-4\text{H-pyran}$, **G11**: alkyl = CH_3) are substrates to illustrate the effect of second sugar moiety. **G10** bears a simple pyranose backbone (all hydroxy groups on Gal unit of **G1** removed). Methyl glycoside **G11** is a monosaccharide that features only a 4-O-methyl glucuronosyl residue.

AMOR Activity of Synthesized Disaccharides

To determine the effect of synthesized disaccharides on the acquisition of competency by the pollen tubes and their response to ovular attraction (AMOR activity), we performed a semi-in vitro *Torenia ovule-manipulating* assay using a glass needle (Fig. 4A; Mizukami et al., 2016). A hand-pollinated pistil (15 mm) is excised and cultivated in a thin layer of agar growth medium containing the synthesized disaccharide. A single ovule excised from a fresh ovary is positioned in front of the pollen tube with a glass needle. The competent pollen tube is attracted to the micropylar end of the embryo sac when placed within $30\ \mu\text{m}$ in front of the pollen tube (Fig. 4A and B), while the noncompetent pollen tube fails to target the ovule placed in front of it (Fig. 4B). It was observed that the pollen tube acquired competency when the growth medium contained $1.35\ \mu\text{M}$ of **G1** (Fig. 4B).

All of the synthesized disaccharides variants were tested for AMOR activity. The disaccharides have been grouped into different categories based on the type of linkage, presence or absence of functional residues, and their arrangement in sugar molecule to make the interpretations of results easier (Fig. 5).

G1 having beta linkage showed a significantly greater percentage of pollen tubes attracted to the ovules than the control (growth media without **G1**) and its alpha anomer **G2** (2.4 times) when included in the assay medium (Fig. 5A; Supplemental Table S1) as reported by Mizukami et al. (2016). Replacement of Gal residue (second saccharide) of **G1** with a similar sugar residue such as Glc (**G3**) did not lead to significant

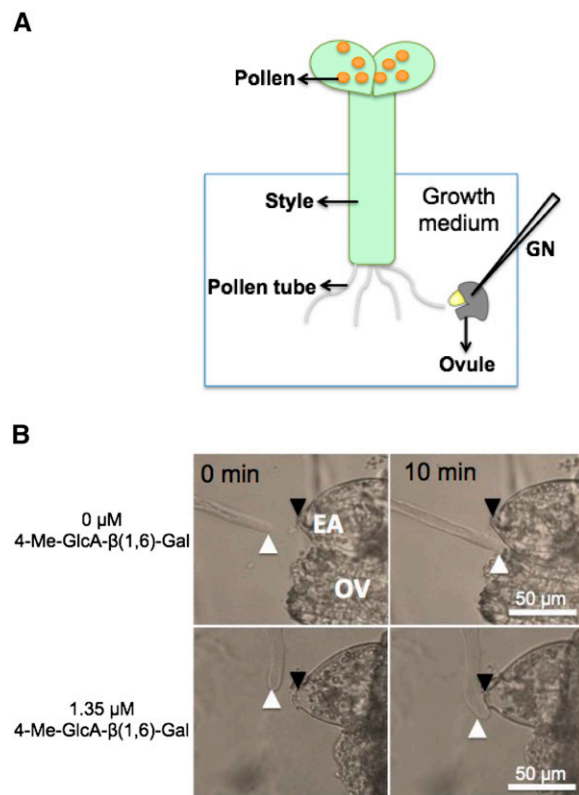


Figure 4. AMOR assay to evaluate the responsiveness of a pollen tube. A, Schematic of the semi-in vitro pollen tube guidance assay for quantification of pollen tube competency (responsiveness) to ovular attraction. As shown, pollen tube growing through the cut style is allowed to grow on a growth medium with/without the disaccharide to examine their response capability by placing a freshly prepared ovule near the tip of an elongating pollen tube using a glass needle (GN). B, AMOR assay showing a pollen tube (white arrowhead) growing away from the opening of egg apparatus (black arrowhead, EA) of an ovule (OV) placed in front of it in absence of AMOR disaccharide (top). In the presence of **G1** ($1.35\ \mu\text{M}$), the pollen tube is attracted toward the opening of the egg apparatus (bottom).

difference in the percentage of pollen tubes being attracted to the ovules (Fig. 5B; Supplemental Table S1). Disaccharide **G4**, with an unusual (1,3)-linkage resulted in a 2-fold drop in percentage of pollen tubes being attracted to the ovules than **G1** (Fig. 6B). A similar result was also observed in the case of disaccharide **G5** having (1,2)-linkage (Fig. 5C). To our great surprise, addition of the pyranose analog **G10** in the assay medium resulted in a greater percentage of pollen tubes being attracted to the ovules, which was comparable with **G1** (Fig. 5B; Supplemental Table S1). However, complete removal of sugar-like structure is detrimental, as inclusion of **G11** in the assay medium resulted in 3.4-fold drop in the percentage of pollen tubes being attracted to the ovules compared to **G1**. 4-O-methyl group in the glucuronosyl residue was found to be essential for AMOR activity.

Elimination of methyl group from the C-4 position of **G6** led to about 24-fold reduction in the percentage of

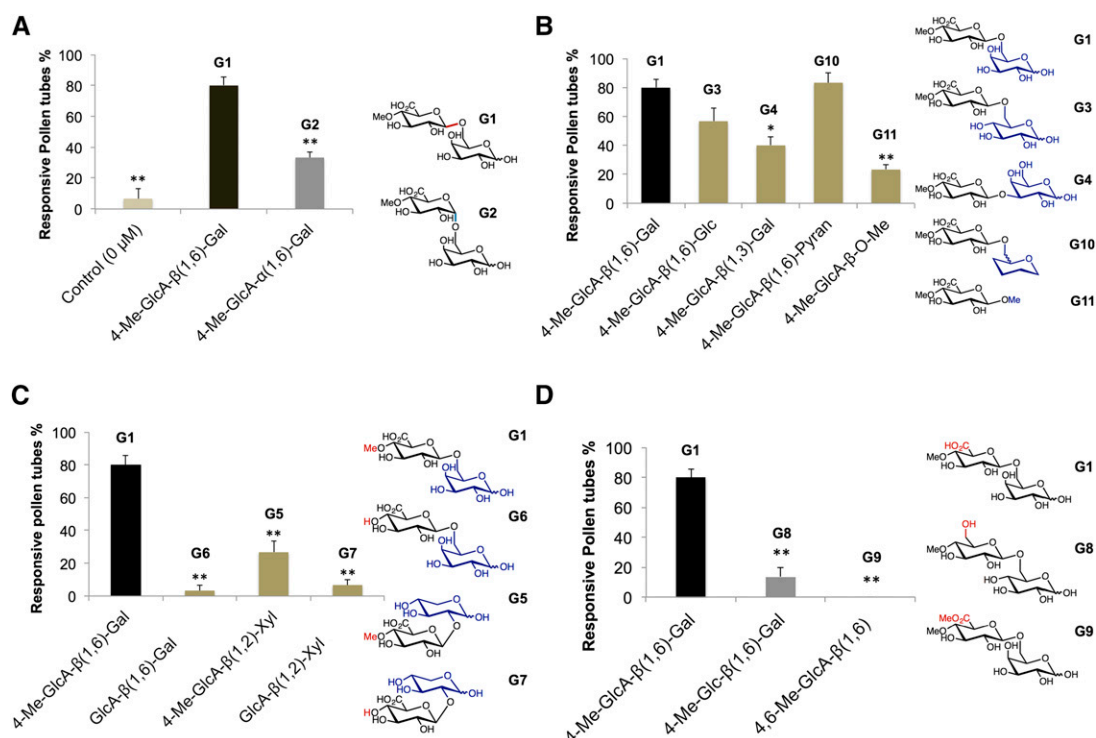


Figure 5. AMOR activity of synthesized analogs. A, AMOR activity of **G1** versus **G2** and the control. B, AMOR activity of **G1** versus **G3** analog bearing a 1,6-linked Glc, **G4** analog bearing a 1,3-linked Gal, **G10** analog with a pyran backbone, and **G11** analog bearing a methyl glycoside. C, AMOR activity of **G1** versus analogs **G6** (lacking 4-*O*-Me) and **G5** versus **G7** (lacking 4-*O*-Me). D, AMOR activity of **G1** versus hydroxyl analog (**G8**) and methyl ester analog (**G9**). Concentration of the synthesized analogs used in the assay is 1.35 μM. Data are means of three independent experiments ($n = 30$ pollen tubes). Bars represent the SEM. All statistical comparisons were made against **G1**. Significant differences were calculated by Student's *t* test, one-way ANOVA with posthoc Tukey HSD test, and Scheffé test (* $P < 0.05$, ** $P < 0.01$).

pollen tubes being attracted to the ovules compared to **G1** (Fig. 5C; Supplemental Table S1) as reported by Mizukami et al. (2016). This result was consistent with that of disaccharides having (1,2)-Xyl linkage (**G5** and **G7**). Removal of 4-*O*-methyl group (**G5**→**G7**) resulted in a 4-fold reduction in the percentage of pollen tubes being attracted to the ovules compared to **G1** (Fig. 5C; Supplemental Table S1). The COOH group in the C-5 position of **G1** was also essential to the functionality of glucuronosyl residue. The addition of hydroxymethyl analog **G8** in the assay medium resulted a 6-fold reduction in the percentage of pollen tubes being attracted to the ovules compared to **G1**, while the methyl ester analog **G9** led to complete loss of competency response, as none of the pollen tubes respond to the nearby ovules in its presence compared to **G1**.

Pollen Germination Activity of Synthesized Disaccharides

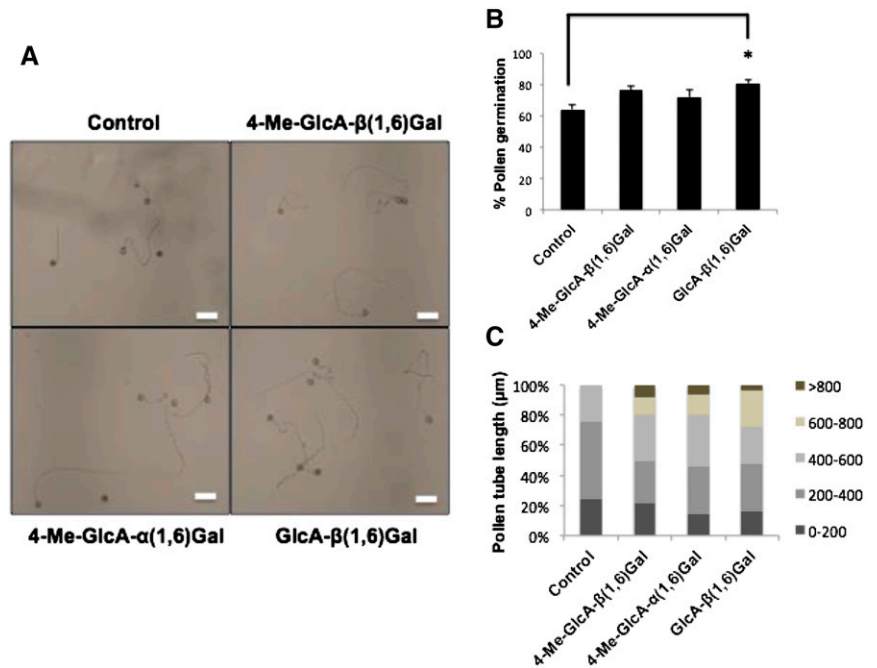
Since AGPs are known to promote pollen germination and pollen tube growth, the disaccharide **G1** and its closest analogs **G2** and **G6** were tested for their ability to induce pollen germination and pollen tube growth (Fig. 6). There was no significant difference in the pollen germination rates among these analogs.

However, pollen germination was significantly improved (1.2-fold) upon germination of pollen in medium containing **G6** compared to the control without synthesized sugars (Fig. 6B). All three synthesized sugars improved the pollen tube growth compared to the control; however, there was no significant difference in pollen tube length between the sugars (Fig. 6, A and C).

DISCUSSION

AGPs play an important role in plant growth and development. The major component of AGPs is the glycan moiety, which often constitutes >90% of the molecule (Knoch et al., 2014). The differences in glycan structure of AGPs could be used as markers in plant development and reproduction (Coimbra et al., 2007; Dilokpimol et al., 2014). The understanding of the functional roles of glycan moiety of AGPs in plants has relied on either the use of β -Yariv reagents that bind to β -1,3-galactan moiety of AGPs (Kitazawa et al., 2013) or monoclonal antibodies that recognize different AGP glycan epitopes (Seifert and Roberts, 2007). Due to the complex and heterogeneous nature of the glycan moieties, it has been difficult to study the structure-function

Figure 6. Torenia pollen germination assay with synthesized sugar variants. A, Picture shows the effect of 4-Me-GlcA- β (1,6)-Gal, 4-Me-GlcA- α (1,6)-Gal and GlcA- β (1,6)-Gal (G6) on germination of Torenia pollen (scale bar = 100 μ m). B, Percentage pollen germination in the presence of 4-Me-GlcA- β (1,6)-Gal, 4-Me-GlcA- α (1,6)-Gal, and GlcA- β (1,6)-Gal in germination medium compared to control without any of these sugars. Concentration of each of the disaccharide used in the germination media is 1.35 μ M. Data are average of six biological replicates. One-way ANOVA with posthoc Tukey HSD test and Bonferroni and Holm tests were used to calculate significant differences and for multiple comparisons (* $P < 0.01$). C, Percentage of pollen with different pollen tube length (μ m) after 2 h of germination in the presence or absence of 4-Me-GlcA- β (1,6)-Gal, 4-Me-GlcA- α (1,6)-Gal, and GlcA- β (1,6)-Gal in the pollen germination medium. Length of 50 pollen tubes was measured for each treatment.



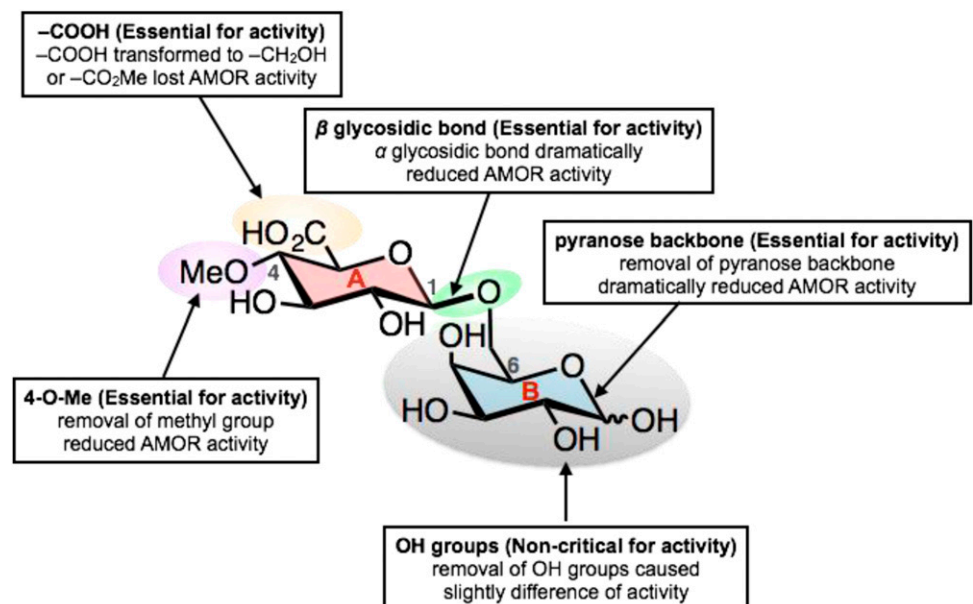
relationship of AGP glycans. In the future, a chemical biology approach together with classical genetics utilizing mutants of enzymes involved in biosynthesis of the glycan moiety of arabinogalactans would enable us to dissect the functional roles of glycan moieties of AGPs.

AGPs are prevalent in the reproductive tissues of plants, and they have been proposed to play prominent roles in plant sexual reproduction (Pereira et al., 2015). Our studies to investigate the process of competency control during pollen tube guidance led to the discovery of an ovular factor AMOR (Arabinogalactan molecule) that induces competency in Torenia pollen

tubes to respond to LURE attractants released from the ovules (Mizukami et al., 2016). It was further revealed that AMOR activity resided in the terminal methyl-glucuronosyl residue of the arabinogalactan (Mizukami et al., 2016) polysaccharide and a chemically synthesized disaccharide, the beta epimer of methyl-glucuronosyl Gal (G1), showed full AMOR activity (Mizukami et al., 2016). Though 4-Me-GlcA-Gal is found on the exposed side chains of AGPs, their functional role has remained elusive.

We carried out an SAR study with the goal to decipher the relationship between various residues present

Figure 7. Effect of structural modification of G1 on AMOR activity in Torenia. The SAR results were concluded and showed clearly of all structural information related to AMOR activity.



on the AMOR disaccharide and their influence on ability of the pollen tubes to respond to ovular guidance cues (competency control). Using a synthetic chemistry approach, we have determined the requirement of disaccharide structure for AMOR activity using **G1** and its analogs (Fig. 7). The studies revealed that the presence of 4-*O*-methyl-glucuronosyl (ring A) is essential for AMOR activity. Both the 4-*O*-methyl and -COOH found on ring A were irreplaceable for activity. The importance of glucuronosyl residue for AMOR activity is also supported by our previous work, where in hydrolytic degradation of the terminal glucuronosyl residues of AGPs with glucuronidase dramatically decreased the AMOR activity (Mizukami et al., 2016).

Stereochemistry of the sugar linkage (β -glycosidic bond) is also an important structural element for the AMOR activity. This is consistent with the fact that β -glycosidic bonds are found naturally between saccharide units of AGPs (Knoch et al., 2014). The pyranose backbone (ring B) of AGPs was also found to be essential for activity, but the surprising discovery was that the simple tetrahydropyran moiety could replace the complex Gal moiety in terms of AMOR activity.

Through this SAR study, we identified the crucial structure of AMOR ligand required to maintain its affinity for the putative receptor. The variation in responses of the pollen tubes to various structural variants of the disaccharide is most likely a result of the differences in affinity of these variants to the putative AMOR receptor. The effect of these structural variants on competency control is presumably dosage dependent, as the presence of higher concentrations of some of the variants in the medium would result in a greater proportion of pollen tubes becoming competent to respond to the guidance cues. The discovery of noncritical residues of the AMOR disaccharide would allow live cell imaging to study AMOR localization, dynamics, and binding to the putative receptor by attaching fluorescent tags to these residues without loss of AMOR activity. The terminal -OH group found on the pyranose backbone of the second sugar would be most suitable for this purpose, as it does not lead to any loss of activity. Use of fluorescent ligands with appropriate imaging properties can also be used to investigate the regulation of putative receptor after activation by the AMOR disaccharide. The use of fluorescent AMOR ligand could reveal the spatial organization of the putative AMOR receptors in the pollen tube and thus aid in their identification.

Arabinogalactan proteins are highly expressed in pollen, and they play an important role in pollen development, germination, and pollen tube growth (Coimbra et al., 2009; Costa et al., 2013; Pereira et al., 2014). Pollen tube growth was severely affected by the Yariv phenyl glycoside, a reagent known to bind specifically to AGPs (Roy et al., 1998; Mollet et al., 2002). Immunolabeling studies using monoclonal antibodies specific to several glycosidic epitopes of AGPs

have revealed distinct distribution of AGPs in the in vitro-germinated pollen tubes of *Nicotiana tabacum* and Arabidopsis, further strengthening their importance in pollen germination and growth (Li et al., 1992; Pereira et al., 2006). The LM2 monoclonal antibody that specifically recognizes the carbohydrate containing epitope of β -linked GlcA produced a ring-like fluorescence around the emerging region of pollen tubes, suggesting a role for GlcA residue in pollen tube growth (Pereira et al., 2006). Thus, we decided to test if Me-GlcA- β (1,6)-Gal and its closest analogs had an effect on pollen germination and pollen tube growth. There was no statistical difference in pollen germination or pollen tube growth in the presence of Me-GlcA- β (1,6)-Gal in the medium compared to control, suggesting that its role was specific to competency control. Interestingly, GlcA- β (1,6)-Gal enhanced pollen germination compared to control, suggesting that the 4-*O*-methyl group in glucuronosyl residue, which is essential for AMOR, may be detrimental to pollen germination. Stereochemistry of linkage (α vs β glycosidic bond) is also not important for pollen germination, as both the anomers of Me-GlcA-(1,6)-Gal displayed similar rates of pollen germination and pollen tube growth. It is of interest to know how biosynthesis of arabinogalactan sugar chain is spatiotemporally regulated in the pistil by β -glucuronosyltransferases (for addition of GlcA to the galactan; Geshi et al., 2013; Knoch et al., 2014) and by unidentified methyltransferases (for addition of the 4-*O*-methyl group to the glucuronosyl galactan).

The identification of the role of 4-Me-GlcA-Gal and the dissection of its critical residues in imparting competency of pollen tubes not only enhance our understanding of the complex fertilization process in flowering plants, but also add to our understanding of plant sugar signaling. The chemical biology approach described in this work provides a great opportunity to gain a molecular-level understanding of these disaccharides and elucidate their functional roles. This SAR study would serve as a guideline for future studies aimed to understand sugar signaling in plant reproduction. This study forms the first step toward understanding the mechanism of AMOR action, including the identification of its putative receptor and the downstream signaling pathways.

MATERIALS AND METHODS

Chemical Synthesis of Disaccharides

See the full experimental details for synthesis of all compounds in the supplemental information. The purity of all synthesized compounds was verified, and pure compounds were used for biological studies.

Plant Materials and Growth Conditions

Torenia fournieri cv 'blue and white' were grown on soil at 28°C with a 16-h photoperiod [approximately 150 $\mu\text{mol}/(\text{m}^{-2} \text{s}^{-1})$]. We used flowers from these plants for the AMOR assay and pollen germination assay.

AMOR Assay

AMOR activity was measured using AMOR assay performed as described previously (Mizukami et al., 2016). A glass-bottom dish (D210402; Matsunami) was covered with modified Nitsch's medium. The center of the agar plate was cut into an approximately 18 × 22-mm² square and the agar block was removed. Growth medium (5-fold modified Nitsch's medium containing 13% polyethylene glycol 4,000 [w/v] and 1% Suc [w/v]) with synthesized saccharides (1.35 μM, 13.5 μM) and 1.5% ultra-low gelling agarose (agarose type IX-A; Sigma) were filled up to 220 μL with 20 mM MES/KOH (pH 6.8) and poured into the square. Before solidification, the cut end of a 15-mm-long hand-pollinated style was embedded at the center of the growth medium. This agar plate was incubated in the dark at 28°C for 14 h. After incubation, water-saturated silicone oil (KF-96-100CS; Shin-Etsu Chemical) was layered to the growth medium and hand-pollinated style. Ovules were dissected from the pistils on the growth medium under observation using a stereomicroscope (SZ61, Olympus). A single ovule was picked up using a glass needle produced with a glass needle puller (PC-10; Narishige) and placed in front of the pollen tubes using manipulator (MMN-1, MMO-202N, MMO-220A, Narishige) under observation using an inverted microscope (IX71, Olympus). Pollen tube response was evaluated based on the attraction of the pollen tube to the micropylar end of the embryo sac. The pollen tube was scored as responsive if, when placed within 30 μm of an ovule, the pollen tube altered its direction of growth and continued growing to make contact with the micropylar end of egg apparatus. The pollen tubes were scored as nonresponsive if they did not alter their direction of growth or did not make contact with the egg apparatus.

Torenia Pollen Germination Assay

Pollen was collected from the anthers of fully open *Torenia* flowers and suspended in a 2-mL microcentrifuge tube containing liquid growth medium (2-folded modified Nitsch's medium containing 13% polyethylene glycol 4,000 [w/v] and 2% Suc [w/v], pH 6.5). The suspended pollen-containing medium was mixed well and split equally into different microcentrifuge tubes with synthesized saccharides (1.35 μM) or without it (control). By this method, the pollen density was kept uniform across the treatments to control the population effect. These microcentrifuge tubes were incubated in dark at 28°C for 2 h. After 2 h, the liquid suspension containing germinated pollen was placed on a microslide and observed under bright field with an Axio Imager2 (Zeiss) microscope. Percentage germination was calculated by counting 100 pollen grains from different fields of microslide. Data were collected from six different biological replicates. Pollen tube length was calculated using the ImageJ software from 50 pollen tubes for each treatment selected at random.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Synthesis of glycosyl donors GD1 and GD2.

Supplemental Figure S2. Synthesis of glycosyl donor GD3.

Supplemental Figure S3. Synthesis of glycosyl acceptor GA1 and GA2.

Supplemental Figure S4. Synthesis of glycosyl acceptor GA3.

Supplemental Figure S5. Synthesis of glycosyl acceptor GA4.

Supplemental Figure S6. Programmable glycosylation of glycosyl donor and acceptor, the yields of glycosylation product G' and the deprotection product G (the total yield of two anomers and ratio of β:α is shown in parenthesis).

Supplemental Figure S7. Synthesis of G9.

Supplemental Figure S8. Synthesis of 4-Me-GlcA-β-OME (G11).

Supplemental Table. AMOR activity results for synthetic AMOR and derivatives.

Supplemental Materials and Methods. Supplemental Materials and Methods.

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