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Identification and characterization of inhibitors of UDP-glucose and UDP-sugar pyrophosphorylases for *in vivo* studies

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SUMMARY

UDP-sugars serve as ultimate precursors in hundreds of glycosylation reactions (e.g. for protein and lipid glycosylation, synthesis of sucrose, cell wall polysaccharides, etc.), underlying an important role of UDP-sugar-producing enzymes in cellular metabolism. However, genetic studies on mechanisms of UDP-sugar formation were frequently hampered by reproductive impairment of the resulting mutants, making it difficult to assess an *in vivo* role of a given enzyme. Here, a chemical library containing 17 500 compounds was separately screened against purified UDP-glucose pyrophosphorylase (UGPase) and UDP-sugar pyrophosphorylase (USPase), both enzymes representing the primary mechanisms of UDP-sugar formation. Several compounds have been identified which, at 50 μM , exerted at least 50% inhibition of the pyrophosphorylase activity. In all cases, both UGPase and USPase activities were inhibited, probably reflecting common structural features of active sites of these enzymes. One of these compounds (cmp #6), a salicylamide derivative, was found as effective inhibitor of *Arabidopsis* pollen germination and *Arabidopsis* cell culture growth. Hit optimization on cmp #6 yielded two analogs (cmp #6D and cmp #6D2), which acted as uncompetitive inhibitors against both UGPase and USPase, and were strong inhibitors in the pollen test, with apparent inhibition constants of less than 1 μM . Their effects on pollen germination were relieved by addition of UDP-glucose and UDP-galactose, suggesting that the inhibitors targeted UDP-sugar formation. The results suggest that cmp #6 and its analogs may represent useful tools to study *in vivo* roles of the pyrophosphorylases, helping to overcome the limitations of genetic approaches.

Keywords: chemical library screening, inhibitors, UDP-sugar synthesis, pyrophosphorylases, pollen germination, *Arabidopsis* cell culture, enzyme kinetics, *Arabidopsis thaliana*, reverse chemical genetics.

INTRODUCTION

UDP-sugars are substrates for hundreds of glycosyltransferases in all organisms, and they serve as direct precursors for synthesis of oligo- and polysaccharides, glycoproteins, glycolipids, and many other glycosylated compounds (Feingold and Avigad, 1980; Kleczkowski *et al.*, 2010; Kotake *et al.*, 2010; Bar-Peled and O'Neill, 2011; Kleczkowski and Decker, 2015). The primary event leading to the formation of a specific UDP-sugar involves the action of a UTP-dependent pyrophosphorylase which 'activates' a given sugar by linking it to a UDP-moiety in a reaction involving UTP and a sugar-1-P. The major pyrophosphorylases include UDP-Glc pyrophosphorylase (UGPase) and UDP-sugar pyrophosphorylase (USPase), both enzymes differing in specificity for sugar-1-P as a substrate, and both located in the cytosol (Kotake *et al.*, 2004,

2007; Litterer *et al.*, 2006a,b; Kleczkowski *et al.*, 2010, 2011a). *Arabidopsis* UGPase was already crystallized (McCoy *et al.*, 2007) and its tertiary structure is generally similar to UGPases from other eukaryotes (Roeben *et al.*, 2006; Steiner *et al.*, 2007; Yu and Zheng, 2012), whereas USPase was only crystallized from *Leishmania major*, a protozoan pathogen (Dickmanns *et al.*, 2011). Despite less than 20% identity between UGPase and USPase proteins, based on amino acid sequences, they both share common structural blueprint, including also common details of their active sites (Dickmanns *et al.*, 2011; Kleczkowski *et al.*, 2011b).

Of the two pyrophosphorylases, UGPase has been by far the most extensively studied, both in plants, animals and bacteria (Knop and Hansen, 1970; Kleczkowski, 1994b;

Bosco *et al.*, 2009). Besides being strongly conserved in plants, with over 70% identity at amino acid level between UGPases from different organisms, the enzyme is regulated by oligomerization, with monomer as the only active form (Martz *et al.*, 2002; Kleczkowski *et al.*, 2005; McCoy *et al.*, 2007; Decker *et al.*, 2012). Plant tissues usually contain two highly homologous isozymes of UGPase, with similar kinetic properties (Meng *et al.*, 2008). Studies with transgenic plants in which expression of one or both UGPase genes was affected have revealed that the enzyme is not rate limiting in primary metabolism (Zrenner *et al.*, 1993; Meng *et al.*, 2009b), but is essential for plant survival, and its loss or drastically decreased activity result in male sterility or a decreased number of seeds produced (Chen *et al.*, 2007; Meng *et al.*, 2009b; Mu *et al.*, 2009). A double *Arabidopsis* mutant with highly impaired growth, where both UGPase genes were 'knocked out' was also reported, but its fertility could be maintained only when grown on an artificial media containing 1.5% UDP-Glc (Park *et al.*, 2010). Transgenic plants overexpressing UGPase have frequently been reported to have modified cellulose content (Coleman *et al.*, 2007; Wang *et al.*, 2011; Li *et al.*, 2014), consistent with the role of the enzyme as a mechanism for providing UDP-Glc for cellulose biosynthesis. UGPase was also implicated in regulation of plant cell death (Chivasa *et al.*, 2013).

In contrast to UGPase, which mainly produces UDP-Glc (Decker *et al.*, 2012), USPase appears central to the formation of a variety of UDP-sugars, e.g. UDP-Glc, UDP-Gal, and UDP-GlcA (Kotake *et al.*, 2004, 2007; Litterer *et al.*, 2006a,b) which are required in a plethora of glycosylation reactions producing many metabolites and structural components of plant cell. The enzyme, coded by a single gene in plants, was unequivocally identified and characterized only recently (Kotake *et al.*, 2004), but the USPase-like activities (e.g. 'UDP-galactose pyrophosphorylase' or 'UDP-xylose pyrophosphorylase') have been reported for at least 50 years now (Kleczkowski *et al.*, 2011a). The role of USPase has been most thoroughly studied in *Arabidopsis*, using loss-of-function 'knockout' plants (Schnurr *et al.*, 2006; Kotake *et al.*, 2007). Whereas these studies were hampered by male sterility of the heterozygous plants, this was partly overcome by mutant complementation and miRNA approaches (Geserick and Tenhaken, 2013a,b). In the latter studies, USPase was implicated as being essential for arabinose and xylose recycling for cell wall synthesis. Similar to studies on UGPase, however, the USPase 'knockout' plants were of a rather limited value in studying exact functions of this protein in plants.

Given difficulties with the use of transgenic plant 'knockouts' in studying *in vivo* roles of UDP-sugar producing pyrophosphorylases, there is a need to supplant these classical reverse-genetics approaches with other method(s). One of such methods, so called reverse chemical genetics, involves effects of an *in vivo* application of a specific

inhibitor of a given enzyme to the living cells/tissues. This approach, if successful, circumvents the need for mutant generation, and allows the use of wild-type (wt) plants (Stockwell, 2000; Blackwell and Zhao, 2003). In the present study, by screening a chemical library, we have identified several compounds which inhibited both UGPase and USPase activities. Some of these compounds also effectively inhibited cell growth and pollen germination in *Arabidopsis*. Hit optimization of one of the inhibitors resulted in compounds which were more effective inhibitors of the pyrophosphorylases, but also had stronger *in vivo* effects. The rationale and advantages for the use of the identified inhibitors are discussed.

RESULTS

Screening for inhibitors and dose-responses

To search for inhibitors of UGPase and USPase, we used chemical library developed in the Chemistry Department at Umeå University. The library contains a collection of about 17 500 compounds, which cover a wide range of chemical structures. As earlier reported (Decker *et al.*, 2014), UTP-dependent pyrophosphorylases are especially well suited for high-throughput chemical screening because they produce pyrophosphate (PPi), which then can be 'coupled' to ATP-sulfurylase and firefly luciferase activities. This results in luminescence which can be easily quantified using a high-throughput setup. The assay system (assay A) allowed for quantitative measurement of UGPase and USPase activities, down to a pmol per min level, and the activities were linear with time and proportional to the amount of the enzyme added (Decker *et al.*, 2014). Also, both enzymes were found to be stable for at least 1 h at room temperature and were insensitive to up to 5% DMSO, which was a component of all inhibitor solutions.

Using the luminescence-based assay, we have separately screened the library for inhibitors of barley UGPase and *Leishmania* USPase, with each compound kept at 50 µM. The primary screen was considered completed when data of acceptable quality ($Z' > 0.5$) (Zhang *et al.*, 1999) had been collected from all compound plates against both target enzymes. The final average and median Z' values for the UGPase screen were 0.79 and 0.80, respectively, while those for the USPase screen were 0.77 and 0.78, respectively (Figure S1a). In the screen, a 70% inhibition cut-off value was set for selecting the most potent inhibitors. The principles of selection of inhibitors that fulfilled this criterion are given in Figure S1(b).

Primary screens for both enzymes resulted in the identification of 181 inhibitors of UGPase and 26 inhibitors of USPase (Figure 1a). Based on the 70% inhibition cut-off criterion, some of those compounds inhibited only UGPase or USPase and some affected both of these enzymes. After secondary screening (re-screening) and counter-screening

Figure 1. Screening the chemical library for UGPase and USPase inhibitors. (a) Outline of the screening campaign. The library was separately screened, using a luminescence-based assay, against purified barley UGPase and *Leishmania major* USPase. Arrows (a–d) point to the number of compounds screened and identified after primary and secondary screenings, and after dose–response.

(b) Summary of dose–response (DR) results and inhibitor effectiveness in other assay systems (assay B and C for forward and reverse reaction, respectively), using barley UGPase. (+) over 50% inhibition at 100 μM of inhibitor; (++) over 50% inhibition at 50 μM of inhibitor.

(identification of luciferase/ATP-sulfurylase inhibitors), a total of 13 compounds remained, all of them inhibiting both UGPase and USPase (Figure 1a). These 13 inhibitors were further validated using two orthogonal assay systems, based on quantification of P_i released from PP_i (forward rxn, assay B), and on spectrophotometric assays of Glc-1-P formed during assays (reverse rxn, assay C), respectively. Those assays narrowed down the number of inhibitors to five compounds, although in some cases their effects were not as efficient as seen after primary and secondary screenings run using assay A (Figure 1b). The reason for this difference in sensitivity between assay A and assay B (both measured forward reaction of the pyrophosphorylases) is unclear, but it is possible that either luciferase or ATP-sulfurylase (assay A) might have been themselves partially inhibited by these compounds. Whereas the luminescence-based assay A was useful to examine effects of thousands of chemicals during high-throughput screening, it was employed just for the purpose of the screening.

The inhibitors were subsequently used in dose–response assays, where they were tested at several concentrations

against UGPase and USPase activities, again using the ATP-sulfurylase/luciferase assay system (assay A). The dose–response approach reconfirmed that each of the five compounds, at 50 μM , caused more than 50% inhibition of targeted enzymes (Figure S2). Interestingly, the five compounds were also inhibitory against purified Arabidopsis USPase and Arabidopsis UGPase1 and UGPase2 isozymes, suggesting that they acted as general inhibitors of the pyrophosphorylases.

The five compounds identified after dose–response study belong to different chemical classes (Figure S2). They included a quinoline (cmp #3), which is a relatively large and multifunctional screening hit, a non-cyclic imide/diacylamine (cmp #6), an enone (cmp #9), a carboxythiourea (cmp #10), and an oxazole (cmp #11). The cmp #6 was purchased as 4H-1,3-benzoxazin-4-one (ChemBridge ID 5306512), but in screening hit quality controls it was found to be in the uncyclized imide form (ZINC 5898067), as shown in Figure S2. All these compounds were then further analysed for their suitability and effects using plant *in vivo* systems. Based on outcomes of those studies, the most promising effector was selected for hit optimization to improve its inhibitory effects on UGPase/USPase activities and during *in vivo* studies (see below).

Identification of cmp #6 as effective *in vivo*

Compounds identified during screening of the chemical library were subsequently analysed for their likelihood of crossing of plant membranes. Lipinski *et al.* (2001) provided several simple criteria for the likelihood of a transmembrane migration and absorption of a compound, when administered orally, by a mammalian tissue. Those criteria include molecular mass of a compound, its lipophilicity, and the number of hydrogen-bond donors and number of hydrogen-bond acceptors. A similar, but modified, set of rules has been designed by Limmer and Burken (2014) for transport of an organic compound into plant roots, with a quantitative estimate of plant translocation (QEPT) providing an integrated measure of the translocation. In Figure 2(a), which represents several plots of a given translocation criterion versus desirability function, the positions of all compounds identified after dose–response study were compared to each other. For five out of six translocation criteria examined, the position of cmp #6 was close to the optimal, in contrast to other compounds, with possible exception of cmp #11. Scaled and weighted integration of all desirability parameters, computed as in Limmer and Burken (2014), resulted in the highest QEPT value (0.22) for cmp #6, whereas for other compounds this value ranged from 0.0003 (cmp #3) to 0.12 (cmp #10) (Figure 2b). Based on those physicochemical predictors, we have identified cmp #6 as, putatively, the most promising candidate inhibitor for *in vivo* studies.

Figure 2. Prediction of translocation of inhibitors of UGPase/USPase into a plant cell, based on their physicochemical properties.

(a) The properties of the inhibitors versus desirability function for each molecular descriptor (Limmer and Burken, 2014). Gray shading marks physicochemical properties that commonly allow translocation; cmp #6 is marked with an arrow.

(b) Scaled and weighted molecular descriptors combined into a quantitative estimate of plant translocation (QEPT), with the most promising inhibitor marked in bold.

The QEPT values are only expressing a likelihood of a given compound crossing a root cell plasma membrane, and they are not a substitute for actual *in vivo* experimental data, especially when using non-root based experimental system (Limmer and Burken, 2014). Thus, in subsequent studies we used all five compounds identified as inhibitors of UGPase/USPase to study their effects on Arabidopsis pollen germination and on the growth of Arabidopsis habituated cell culture. The two processes heavily depend on provision of UDP-sugars, required both for the

growth of a pollen tube (Litterer *et al.*, 2006b) and for mitotic cell division to produce new cell walls for daughter cells (Kunz *et al.*, 2014). In the pollen test, both pollen germination rate and pollen tube growth were scored after 6 h of the inhibitor treatment, with each inhibitor applied at 25 μM (Figure 3a, b). The results indicated that only cmp #6 and #9 had strong effect on both scored parameters, whereas other compounds were ineffective. When a dose-response of cmp #6 on pollen germination was studied, using a 50 nM to 5 μM range of cmp #6, the apparent half

maximal inhibitory concentration (IC_{50}) value was estimated at ca. 2 μ M (Figure 3c).

For effects on cell culture, the inhibitors were used at 50 μ M, and we measured both cell viability and cell mass after 7 days of culture. In both cases, the treatment with cmp #6 was by far most effective, resulting in very few viable cells (Figure 4a), and it drastically reduced the mass of cells produced (Figure 4b). Among other inhibitors, only cmp #11 affected both cell viability and cell mass, but its effect was much weaker than that of cmp #6. Whereas cmp #9 and #10 had some effect on cell amount, they did not affect cell viability. A dose-response study with cmp #6 (Figure 4c) revealed that the cell viability drastically decreased between 2.5 and 25 μ M, indicating that half of the lethal dose (LD_{50}) value is somewhere between those concentrations.

Overall, the results of *in vivo* studies, using two different plant systems, revealed that cmp #6 was the only compound that was effective both in pollen germination test and in cell culture studies. Compound #9, even though serving as effective inhibitor in the pollen test (Figure 3a, b), had no effect on cell viability (Figure 4a) and had low translocation probability, based on QEPT analyses (Figure 2b). This was in contrast with cmp #6 which, based on QEPT, was the most suitable (among compounds tested) as an *in vivo* effector. Thus, from then on, we have used cmp #6 in chemical optimization analyses and for *in vivo* studies.

Hit expansion/optimization for cmp #6

Compound #6 is a non-cyclic imide/diacylamine composed of the aromatic moieties of *p*-chlorobenzoic acid and salicylic acid (Figure S2). In search for analogs of cmp #6, we ordered several related commercially available compounds, which had substitutions to its rings and/or with modifications in its linker region. Thus, the analogs showed variations in the salicyloyl moiety (cmp #6E, #6G, #6H, and #6J), the *p*-chlorobenzoyl moiety (cmp #6A-K) and in the linker region (cmp #6A-E and #6I). (Figure 5).

The analogs of cmp #6 were then tested against UGPase and USPase activities (Figure 6). Since our interests are in studying the functions of plant UDP-sugar producing pyrophosphorylases, in this and subsequent studies we used purified USPase from Arabidopsis (along with barley UGPase) rather than *Leishmania* USPase which we had used to screen the chemical library. Among analogs, the strongest effect, when compared to effects of cmp #6, was observed for cmp #6D and, to some extent, cmp #6I, for both UGPase and USPase activities (Figure 6). Only UGPase activity was affected by cmp #6B and, probably, #6H. Other analogs of cmp #6 had no significant effect on UGPase and USPase activities. The observed inhibition of UGPase, but not USPase, by cmp #6B and #6H was interesting, since they were the only inhibitors that came out in

Figure 3. Effects of inhibitors of UGPase/USPase on Arabidopsis pollen germination and pollen tube growth.

(a) Percentage of germinated Arabidopsis pollen after 6 h on germination media in the presence of 25 μ M inhibitor.

(b) Pollen tube length after 6 h on germination media in the presence of 25 μ M inhibitor.

(c) Effects of cmp #6 at different concentrations on Arabidopsis pollen germination after 8 h incubation. Control refers to DMSO effects. Bars represent means $\pm$ standard deviation (SD), $n = 2$. *Statistical significance according to Student's *t*-test ($P < 0.05$).

our screens which discriminated between UGPase and USPase activities.

Overall, the results suggested that modification of the imide linker in cmp #6 with $CH=CH$ -elongation yielded compounds (especially #6D) which were more effective inhibitors of the pyrophosphorylases than cmp #6

Figure 4. Effects of inhibitors of UGPase/USPase on viability and density of *Arabidopsis* cell culture.

(a) Percentage of living cells observed after 7 days in the presence of 50 μM inhibitor. (b) Mass of cells present after 7 days of growth in the presence of 50 μM inhibitor. Inset shows standard curve used to determine the amount of cells. (c) Effects of cmp #6 at different concentrations on *Arabidopsis* cell viability. Control refers to DMSO effects. Bars represent means ± standard deviation (SD), $n = 3$.

(Figures 5 and 6). Substituents in the *para*-position of the original *p*-chlorobenzoyl moiety were beneficial or well tolerated in the compounds with CH=CH -linker elongation. The beneficial *para*-substituents were all electron donating, although at this point it is difficult to say whether the beneficial effect is due to electronic or steric effects. The inhibitory effect does not seem to stem simply from Michael

acceptor reactivity, since the electrophilic moiety is present in both active and inactive compounds. For the same reason the activity appears not to be due to potential metal chelation motifs.

In subsequent hit optimization studies, we used four commercially available analogs of cmp #6D. Compounds #6D2 and #6D4 contained variations of the *para*-substituent of the cinnamoyl moiety, while #6D1 and #6D3 were non-phenolic amides (Figure 5). When they were all tested against barley UGPase activity, the strongest effect was exerted by cmp #6D2 which, at 50 μM, was about twice as effective as cmp #6D (Figure 7). Other compounds either had no effect or had similar effects as cmp #6D (cmp #6D1 and #6D4) or appeared even to slightly stimulate UGPase activity (cmp #6D3). Effects of cmp #6, #6D and #6D2 were also compared in a dose-response analysis for UGPase (Figure S3), where again cmp #6D2 was stronger inhibitor (IC_{50} of 50 μM) than cmp #6D (IC_{50} of 80 μM) and cmp #6 (IC_{50} of 300 μM). Please note that for this analysis the assays were carried out in the reverse direction (assay C), which was less sensitive to cmp #6 (see Figure 1b) and which explains relatively high IC_{50} values.

Compared to cmp #6D, cmp #6D2 has an *S*-methyl rather than *O*-methyl substituent (Figure 5). Given that cmp #6D2 had stronger inhibitory effect than cmp #6D, this suggested that a slightly larger and more hydrophobic methylthioester group of cmp #6D2 was beneficial for the inhibition. This modification could contribute to a larger surface area and increase van der Waals interactions and/or exclude more unfavored water molecules from the protein surface.

Certainly more studies with other analogs are required for further optimization of cmp #6 derivatives as effective inhibitors of UGPase and USPase activities. At present, however, the results strongly suggest that compounds with free phenols and *para*-substituted cinnamoyl moiety are beneficial for inhibiting those enzymes. The curious exception is cmp #6H (Figure 5), which may have a different binding mode for the inhibition.

Kinetics of UGPase/USPase with cmp #6D2 and cmp #6D

Kinetic analyses of the effects of cmp #6D2, using Dixon plots, resulted in a series of parallel or near-parallel lines both for UGPase and USPase (Figure 8). This suggested an uncompetitive or near-uncompetitive mode of inhibition, consistent with the inhibitor binding only to the enzyme-substrate complex, but not to free enzyme (Segel, 1975). The effect was seen regardless of whether UTP or Glc-1-P was used as a varied substrate. Based on the Dixon plot data and using a method by Cornish-Bowden (1974), it was possible to estimate a K'_i value, corresponding to a dissociation constant for an enzyme-substrate complex bound uncompetitive inhibitor. For UGPase, the K'_i values for cmp #6D2 were 32 and 37 μM versus either UTP or Glc-1-P,

Figure 5. Chemical structures of cmp #6 and its analogs, following two rounds of optimization.

Active compounds were those which, at 50 μM , showed significant inhibition of UGPase or USPase (optimization 1, see Figure 6) and those which showed significant inhibition of UGPase (optimization 2, see Figure 7). Statistical significance according to Student's *t*-test ($P < 0.05$). The compounds can be identified by their respective ZINC or ChemBridge ID numbers: #6 (ZINC 5898067), #6A (CID 5484914), #6B (CID 6558333), #6C (CID 7264936), #6D (CID 6526371), #6E (CID 6527441), #6F (CID 5350337), #6G (CID 6038246), #6H (CID 6033001), #6I (CID 5542787), #6J (CID 6177195), #6K (CID 6233041), #6D1 (CID 5524411), #6D2 (CID 7239769), #6D3 (CID 5128214), and #6D4 (CID 6546423). [Colour figure can be viewed at wileyonlinelibrary.com].

respectively, whereas the respective values for USPase were 72 and 48 μM (Figure 8).

Besides kinetic characterization of the effects of cmp #6D2, we have also studied kinetics of its analog, cmp #6D, versus UGPase. Also in this case, parallel or near-parallel patterns were obtained, suggesting that cmp #6D serves as an uncompetitive inhibitor (Figure S4). The estimated K_i' values for cmp #6D were 50 and 58 μM versus either UTP or Glc-1-P, respectively.

Effects of cmp #6D and cmp #6D2 on selected enzymes

Besides UGPase and USPase, we have also tested some selected enzymes for their response to cmp #6D and #6D2 (Figure S5). These included invertase (a glycosidase), hexokinase (HXK) (a kinase) as well as Glc-6-P dehydrogenase (G6PDH) (a dehydrogenase). These enzymes must have binding pockets for a nucleotide (HXK and G6PDH) and for glucose (all three). None of these enzymes were affected by the inhibitors.

Effects of analogs of cmp #6 on pollen germination

Of all compounds identified after chemical library screening, cmp #6 was found as the most effective *in vivo* inhibitor both during pollen germination tests (Figure 3) and when monitoring growth of cell culture (Figure 4). Since the effects of inhibitors on cell culture required long treatments (7 days) and relatively large volumes of compounds tested, in subsequent *in vivo* studies with analogs of cmp #6 we used only the pollen test. We found this test as very useful, since it represented a convenient and quick (4–8 h) measure of the inhibitor effect and it could be scaled down to small volumes of the media used. In Figure 9, we have compared dose–response effects of cmp #6 and its analogs versus germination rate after incubation with the pollen for 4 h. This allowed for estimation of apparent IC_{50} values for those inhibitors. Both cmp #6D and cmp #6D2 had the IC_{50} values of slightly below 1 μM , and their effect was about two- to three-fold stronger than cmp #6 (IC_{50} of 2.2 μM).

Figure 6. Effects of analogs of cmp #6 on activities of barley UGPase and Arabidopsis USPase.

Assay B was used, where substrate concentrations of Glc-1-P and UTP were kept at K_m values for the UGPase (0.33 mM Glc-1-P and 0.25 mM UTP) (Decker *et al.*, 2012) and USPase (0.23 mM Glc-1-P and 0.077 mM UTP) (Kotake *et al.*, 2007), and inhibitors were at 50 μ M. Control refers to DMSO effects. Bars represent means $\pm$ standard deviation (SD), $n = 2$. *Statistical significance according to Student's *t*-test ($P < 0.05$).

Figure 7. Effects of analogs of cmp #6D on activity of barley UGPase.

Assay B was used, where substrate concentrations of Glc-1-P and UTP were kept at their K_m values (0.33 mM Glc-1-P and 0.25 UTP) (Decker *et al.*, 2012) and inhibitors were at 50 μ M. Control refers to DMSO effects. Bars represent means $\pm$ standard deviation (SD), $n = 2$. *Statistical significance according to Student's *t*-test ($P < 0.05$).

This was consistent with effects of those compounds on USPase and/or UGPase activities (Figures 6 and 7). The estimated IC_{50} for cmp #6 was similar to that obtained for this compound after 6 h treatment (Figure 3c), suggesting that, within the 4 h and 6 h incubation periods, the length of the exposure to the inhibitor had no effect on its IC_{50} characteristic.

In subsequent studies, we concentrated on effects of cmp #6D2, as this was the compound that was the

Figure 8. Dixon plots of the kinetics of barley UGPase (a, b) and Arabidopsis USPase (c, d) with cmp #6D2.

The K_i' values were calculated according to Cornish-Bowden (1974).

strongest inhibitor of UGPase (Figure 7). At a concentration as low as 5 μ M, cmp #6D2 inhibited pollen germination by about 75% (Figure 10). Importantly, the inhibition could

Figure 9. Effects of cmp #6, #6D and #6D2 on Arabidopsis pollen germination. The inhibitors were applied at 0.05, 0.5 and 5 μ M. The resulting plots allowed for determination of IC_{50} values for each inhibitor. Incubation was carried out for 4 h.

Figure 10. Effects of cmp #6D2 and UDP-sugars on Arabidopsis pollen germination. Control refers to 0.05% DMSO (v/v), whereas cmp #6D2 and UDP-Glc were supplemented at 5 μ M and 0.5 mM, respectively. Incubation was carried out for 4 h. Bars represent means $\pm$ standard deviation (SD) of four biological repeats. *Statistical significance according to Student's *t*-test ($P < 0.05$).

be prevented by including both the inhibitor and UDP-Glc or UDP-Gal (but not UDP-GlcA) to the incubation mixture, strongly suggesting that it is the UDP-Glc and UDP-Gal formation, which are affected by the inhibitor. UDP-Glc or UDP-Gal alone (at 0.5 mM) had little or no effect on the rate of pollen germination (Figure 10). UDP-Glc is metabolically linked to many other UDP-sugars which otherwise need to be produced by USPase, e.g. to UDP-Gal via an epimerase or to UDP-GlcA via UDP-Glc dehydrogenase (Reiter, 2008; Bar-Peled and O'Neill, 2011; Kleczkowski and Decker, 2015). Whereas the epimerase reaction is fully reversible, UDP-Glc dehydrogenase is irreversible toward UDP-GlcA formation, and there is no other apparent mechanism allowing for regeneration of UDP-Glc (or UDP-Gal) from UDP-GlcA (Bar-Peled and O'Neill, 2011). This could explain as to why inhibition of germinating pollen was compensated for by provision of UDP-Glc or UDP-Gal, but not

UDP-GlcA (Figure 10). In addition, UDP-Glc dehydrogenase has apparently a less important role in contributing to pollen tube growth (Geserick and Tenhaken, 2013a).

Pollen germination test was also applied to those analogs of cmp #6, which had no effect on activities of UGPase/USPase. Among the selected compounds, cmp #6C, #6E and #6D1 had no effect, whereas both cmp #6A and #6K significantly decreased pollen germination (Figure S6). However, the inhibition was not prevented by UDP-Glc, suggesting that those compounds affected some other mechanism(s) involved in pollen germination, distinct from the pyrophosphorylases.

Effects of salicylic acid and *p*-coumaric acid on pollen germination

Two distinct parts of the chemical structure of cmp #6D2 resemble those of salicylic acid (SA) and *p*-coumaric acid (PA) (Figure S7), both representing important secondary metabolites derived from shikimate pathway. SA is a plant growth regulator, which is involved in plant defense responses and flower thermoregulation, among other functions (Chen *et al.*, 2009), whereas PA is a precursor to a variety of compounds, including simple phenyl propanoids, coumarins and lignin, among others (O'Connor, 2015). In order to test whether *in vivo* effects of cmp #6D2 resemble those of SA and PA, their effects were compared using the pollen test. Addition of either SA or PA did not affect pollen germination, and had no effect on inhibition by cmp #6D2 (Figure S7). Also, SA and PA had no effect on activity of purified UGPase (Figure S7). Finally, addition of cmp #6D2 (with or without SA) to leaves of transgenic Arabidopsis plants containing a GUS construct under a SA-inducible promoter had no effect on SA-dependent induction (Figure S7). The results strongly suggest that both benzoyl rings (resembling SA and PA) are required for cmp #6D2 activity *in vivo*, and that cmp #6D2 effects were not connected to SA regulation/signaling.

DISCUSSION

Classical reverse-genetics studies to elucidate *in vivo* functions of UDP-sugar producing pyrophosphorylases have frequently been hampered by the fact that the loss-of-function mutants were impaired in their reproductive abilities (e.g. Schnurr *et al.*, 2006; Kotake *et al.*, 2007; Meng *et al.*, 2009b; Park *et al.*, 2010; Geserick and Tenhaken, 2013a). In addition, other genes coding for related proteins (e.g. USPase in a UGPase mutant) could compensate for silencing of a given pyrophosphorylase (Meng *et al.*, 2009b). The reproductive abilities of transgenic plants were also compromised in studies of mutants of some other enzymes involved in nucleotide sugar synthesis, e.g. UDP-*N*-acetylglucosamine pyrophosphorylase (Chen *et al.*, 2014) and CMP-3-deoxy-D-manno-octulosonic acid synthetase (Kobayashi *et al.*, 2011). Whereas all these studies

underlined the importance of nucleotide sugars for plant reproduction, other more refined approaches are needed to define physiological roles of the UDP-sugar producing pyrophosphorylases. One alternative, so called 'reverse chemical-genetic' approach (Blackwell and Zhao, 2003), involves the use of inhibitors specifically affecting a given enzymatic activity *in vivo*. The rationale for that has some parallels to the use of 'knockouts' in classical genetics; however, it allows to use wt organisms and to use inhibitors for a temporal and spatial perturbation of the normal wt phenotype. The effects of inhibitors *in vivo* are often very rapid and reversible, allowing for fine-tuning of protein function.

In an effort to identify inhibitors of UDP-sugar producing pyrophosphorylases, we have screened a chemical library, containing 17 500 compounds, using purified barley UGPase and *Leishmania* USPase as targets. The screens identified five compounds which inhibited both UGPase and USPase activities (Figures 1 and S2). As the screenings for inhibitors were done independently from each other, the fact that in each case we ended up with the same five compounds was significant, most probably reflecting the presence of common structural details at the active sites of both enzymes. Whereas UGPases and USPases, based on their overall amino acid sequences, share less than 20% identity, the architecture of their active sites and the positioning of substrate binding domains are generally similar (Kleczkowski *et al.*, 2011b). It is still surprising though, given that UGPase reacts only with Glc-1-P, and to some extent Gal-1-P (Decker *et al.*, 2012; Ebrecht *et al.*, 2015), whereas USPase can easily accommodate those and several other sugar-1-phosphates as substrates (Kotake *et al.*, 2004, 2007; Litterer *et al.*, 2006a; Damerow *et al.*, 2010; Yang and Bar-Peled, 2010). The identified five inhibitors of barley UGPase and *Leishmania* USPase were also effective against activities of purified Arabidopsis USPase and two isozymes of Arabidopsis UGPase (UGPase1 and UGPase2). This again could be rationalized by the fact that, even though *Leishmania* and plant USPases share only ca. 30–35% overall identity at amino acid sequence level, they have common features in their active sites (Yang and Bar-Peled, 2010; Kleczkowski *et al.*, 2011b). The same concerns UGPases from a variety of species, which have highly conserved active site details (Geisler *et al.*, 2004; Roeben *et al.*, 2006; McCoy *et al.*, 2007; Meng *et al.*, 2009a; Kleczkowski *et al.*, 2011b; Yu and Zheng, 2012).

Among the five compounds identified by chemical library screening (Figure S2), cmp #6 appeared as the most promising for *in vivo* analyses. This was based on its physicochemical properties, favoring uptake across plant membranes (Figure 2), but also based on its strong inhibitory effects during Arabidopsis pollen germination tests and on growth of Arabidopsis cell culture (Figures 3 and 4). Hit optimization of cmp #6 resulted in two analogs (cmp

#6D and #6D2), which exerted stronger inhibition than cmp #6 (Figures 6 and 7) and acted as uncompetitive inhibitors of both UGPase and USPase (Figures 8 and S4). Uncompetitive inhibitors are particularly useful during *in vivo* studies since they affect both V_{max} and K_m parameters of their target enzymes, and their effects cannot be eliminated by increasing substrate concentration (Kleczkowski, 1994a). One of the advantages of the inhibitor approach to study protein function is that a single inhibitor when applied to cells or tissues may affect all members of a protein family, and thus prevent problems associated with gene redundancy, and is likely to affect the same enzyme or group of enzymes in different organisms (Kleczkowski, 1994a). These principles were likely involved in our studies with cmp #6 and its analogs, which were effective in pollen test (Figures 3, 9 and 10) and cell culture (only cmp #6 tested) (Figure 4), and their respective effects *in vivo* were roughly proportional to effects on purified enzymes (Figures 6 and 7).

The specificity of the inhibitor effects was tested by applying cmp #6D2 together with UDP-Glc or UDP-Gal. In those cases, no inhibition was observed (Figure 10). This suggested that UDP-Glc and UDP-Gal bypass the inhibitor effects, and was consistent with the inhibitor targeting UDP-Glc/Gal production. It should be emphasized that both UDP-Glc and UDP-Gal can be interconverted by a specific epimerase and that a variety of different UDP-sugars can be derived from UDP-Glc via enzymes other than UGPase and USPase (Bar-Peled and O'Neill, 2011; Kleczkowski and Decker, 2015). As UGPase and USPase are the primary mechanisms producing UDP-sugars in plants, and involved in processes linked e.g. to cell wall synthesis, their inhibition should affect many facets of growth and development of plants (Kleczkowski and Decker, 2015).

The use of inhibitors can obviously be hampered if the inhibitor is not absolutely specific for a given protein target or targets, but also if it is not transported into immediate vicinity of the protein, or if it is degraded/inactivated before it reaches its target(s). On the other hand, the results were similar using separately different inhibitors of UGPase/USPase, as when testing effects of cmp #6 and cmp #9 on pollen (Figure 3). Among analogs of cmp #6 which were not affecting UGPase and USPase activities (Figures 6 and 7), we found some (cmp #6C, #6E and #6D1) which had no effect on pollen germination, and some (cmp #6A, #6K) which did affect the germination (Figure S6). However, in the latter case, the addition of UDP-Glc did not bypass the inhibitor effects, suggesting secondary targets.

Up till now there have been no inhibitors reported for either of the plant UDP-sugar producing pyrophosphorylases. UGPase was found as sensitive to product inhibition, but the reported inhibition constant values with UDP-Glc or PPI were usually relatively high (above 0.1 mM) (Feingold and Avigad, 1980; Kleczkowski, 1994b). Rather,

UGPase regulation predominantly occurs at a transcriptional level via metabolite- or environment-mediated changes in gene expression, leading eventually to changes in UGPase protein content (Ciereszko *et al.*, 2005; Meng *et al.*, 2007, 2009b). Regulation of UGPase may also occur via changes in its oligomerization status, with only monomeric form of plant and *Leishmania* UGPase being active (Martz *et al.*, 2002; McCoy *et al.*, 2007; Dickmanns *et al.*, 2011). The oligomerization is regulated by subtle changes in hydrophobicity of an immediate environment, protein crowding, and the availability of substrates of UGPase (Kleczkowski *et al.*, 2005, 2011b; Decker *et al.*, 2012). Other levels of regulation, although still to be explored in more detail, involve redox modification (Soares *et al.*, 2014; Ebrecht *et al.*, 2015) and phosphorylation (Soares *et al.*, 2014). For USPase, there have been fewer reports on its regulation, but this could be related to the relative 'novelty' of this protein, first time identified in 2004 (Kotake *et al.*, 2004). An interesting observation was that USPase activity correlated with the activity of GlcA kinase, which produces GlcA-1-P, a substrate of USPase. However, it was unclear whether this reflects regulation at protein or gene expression levels (Geserick and Tenhaken, 2013b).

Whereas more studies are required, e.g. determining exact details of the inhibitor-enzyme interaction to guide further optimization of the structure of cmp #6D2, the results demonstrated that cmp #6 and its analogs may be useful in studies on the roles of UGPase and USPase *in vivo*. The IC_{50} value for cmp #6D2 in the pollen germination test (less than $1 \mu M$; Figure 9) appears low enough to use this compound in this and other plant-based systems. On the other hand, there is certainly room for further optimization of its chemical structure, so it can be even more effective in inhibiting UGPase/USPase activities. This hit optimization approach, however, should be carefully balanced, so as not to further compromise the ability of the optimized compound to enter plant cells. It should be also added that we do not know the exact reason for stronger *in vivo* effects of the inhibitors when compared with their effects on purified enzymes. Obviously, in living cells, conditions for any of the enzymes could be more optimal than in the assay mixture. Local factors, such as metabolite channelling, metabolite levels, interactions with other proteins, some unknown regulatory factors, they all could enhance effects of the inhibitors, contributing to the low IC_{50} values observed for *in vivo* conditions.

Also, more efforts should be directed toward search for inhibitors which are specific for a given pyrophosphorylase. In this study, we identified cmp #6B and #6H (Figure 5) which inhibited UGPase, but not USPase, activity, but we did not identify any inhibitor which would be effective against USPase, but not UGPase. Such a compound, at least theoretically, could have a pharmaceutical potential. Whereas UGPase is present in all organisms, USPase

is restricted to plants, some bacteria and protozoans, and not present in humans and other mammals (Yang and Bar-Peled, 2010; Kleczkowski *et al.*, 2011a). This raises the possibility that USPase-specific inhibitors might serve as effective drugs for any pathogenic bacterial or protozoan species that have their own USPase (Kleczkowski and Decker, 2015).

Finally, there is a question of specificity of the inhibitor effects during pollen germination tests. At present we cannot exclude the possibility that these *in vivo* effects are also the result of inhibition of one or more targets that are distinct from UGPase and USPase. The results presented here, especially the observed correlation between the effects of cmp #6D and #6D2 on purified UGPase/USPase activities and their effects during pollen germination test – the stronger the effect of a given inhibitor on purified UGPase/USPase, the stronger its *in vivo* effect – are consistent with both of these enzymes being the targets. The fact that UDP-Glc and UDP-Gal could reverse the inhibition is also pointing toward the inhibitors affecting UDP-Glc/UDP-Gal metabolism. However, at least theoretically, a similar effect could be expected if, for example, one or more of glycosyltransferases activities, which are downstream from UGPase/USPase reactions and which use a given UDP-sugar as a substrate, were also affected by a given inhibitor. In this case, the inhibitor would have to act competitively versus a given UDP-sugar to account for recovery of pollen germination upon provision of the UDP-sugar. On the other hand, chemical structures of UDP-sugars differ from those of cmp #6D and #6D2 (Figure 5), which makes it rather unlikely that they would compete for the same binding pocket on a target protein, although we cannot exclude such a possibility. Those and other considerations can be only properly addressed with more studies, especially those involving metabolomics to assess changes *in vivo* in metabolite profiles and fluxes caused by inhibitor application.

EXPERIMENTAL PROCEDURES

Purified UGPases and USPases

Barley UGPase and *Arabidopsis thaliana* UGPase1 and UGPase2, as well as *Leishmania major* USPase were overexpressed in *E. coli* and purified to homogeneity as previously described (Martz *et al.*, 2002; Meng *et al.*, 2008; Decker *et al.*, 2014). *Arabidopsis* USPase was overexpressed in *E. coli*, using a construct containing coding region of AtUSPase linked to thioredoxin (TRX) (Figure S8). After purification of the overexpressed fusion protein on a Co^{2+} Talon column (Clontech, USA), the TRX part of the recombinant protein was cleaved off with TEV protease (Esposito and Chatterjee, 2006). As both TXR and TEV protease contained a poly-His tag, they were subsequently removed from AtUSPase by passing the proteins through a second Co^{2+} Talon column. Analysis of purified AtUSPase following SDS-PAGE revealed that the protein was essentially homogenous (Figure S8). The purified UGPases and USPases were aliquoted and stored at $-80^{\circ}C$.

Assays of UGPase and USPase

A miniaturized luminescence-based assay (assay A) (Decker *et al.*, 2014) suitable for high-throughput applications was used for the pilot study, primary and secondary screening as well as the dose-response analyses. In the assay, production of PPi by the pyrophosphorylases was 'coupled' to the sulfurylase/luciferase system, the latter producing luminescent light. The standard assay mixture, in a final volume of 15 μ l, contained 100 mM Tris (pH 7.5), 5 mM Mg-acetate, 0.1% BSA, 5 μ M adenosine 5'-phosphosulfate (APS), 1 mM dithiothreitol (DTT), 0.2 mM UTP, an aliquot of glucose-1-P (Glc-1-P), 0.1 mU ATP-sulfurylase (New England Biolabs, USA), 1.5 μ l 10 $\times$ SL reagent (prepared according to manufacturer's instruction, BioThema AB, Handen, Sweden), containing luciferin and luciferase and an aliquot of the target enzyme that was within the linear range of the assay (Decker *et al.*, 2014). Each assay was prepared by combining 5 μ l of pre-assay mixture (as described above but at 3 $\times$ the indicated concentrations, and without Glc-1-P), 5 μ l compound mixture (prepared for final assay concentration: 0.05% Tween-20, 1% DMSO and a compound to be examined) and finally initiated with 5 μ l of glucose-1-phosphate (Glc-1-P) (for final assay concentration of 0.33 mM or 2 mM for UGPase and USPase respectively). Assays were carried out in white polystyrene 384-well plates (Corning, USA).

For each target enzyme, a 3 $\times$ pre-assay mixture plate and 3x Glc-1-P plate were prepared using a multi-channel pipette. The 3 $\times$ compound plate (with inhibitors) was prepared using a Matrix WellMate (Thermo Fisher Scientific, USA) in combination with a Biomek NX (Beckman Coulter, USA) pipetting robot using a 384 tips head, by transferring solution from a compound storage plate into the 3x compound plate. The pipetting robot was also used to semi-automatize the preparations of the assay plates. The assays were analysed using a Wallac 1420 Multilable counter (Perkin Elmer) with Stacker (reads after 9 and 54 min). Each assay plate contained 48 negative controls (standard mixture and DMSO but no compound), 16 positive controls (standard mixture and DMSO but no enzyme or substrate) and eight maximal signal controls (standard mixture and DMSO but with 10 $\times$ target-enzyme loaded). The quality of each assay plate was evaluated using Z' score, calculated using the positive and negative controls as described in Zhang *et al.* (1999). The chemical library screening campaign was initiated when plates with Z' > 0.5 could be routinely produced (Figure S1a). Production of plates with low Z' (according to Chi2 test) generally coincided with clogging of pipette tips.

Another assay (assay B) was based on quantification of the Pi released from inorganic pyrophosphate (PPi), the latter representing product of the pyrophosphorylase reaction. Procedures followed generally those described by Decker *et al.* (2012). Assays (final volume of 50 μ l each) were run on 96-well plates (Sarstedt, Germany) and contained 100 mM HEPES (pH 7.5), 5 mM MgCl₂, an aliquot of UGPase or USPase, and varied concentrations of Glc-1-P and UTP. Reactions were initiated by addition of UGPase or USPase, and were run at room temperature for 12 min. After terminating the reaction by heating at 95°C for 5 min, followed by cooling on ice, 0.5 units of inorganic pyrophosphatase (Roche, Switzerland) per assay were added and the reactions allowed to continue at room temperature for 5 min. The content of Pi was determined by modification of the Fiske-Subbarow method (Aoyama *et al.*, 2001).

Pyrophosphorolytic activities of UGPase and USPase (assay C) were determined, using UDP-Glc and PPi as substrates, and the reaction was assayed spectrophotometrically (at 340 nm) by

coupling the production of Glc-1-P to NADPH formation in the presence of NADP, phosphoglucomutase (PGM) and G6PDH. Assays (each in a final volume of 100 μ l and run on 96-well plates) contained 100 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.3 mM NADP, 5 units each of PGM and G6PDH (both from Roche), varied concentrations of UDP-Glc and PPi, and an aliquot of purified UGPase or USPase. The reactions were initiated with UDP-Glc and run for 5 min at room temperature.

Prior to all assays, stocks of purified UGPase and USPase were diluted from 100- to 10 000-fold, depending on assay system, in 100 mM HEPES (pH 7.5) and 1 mg ml⁻¹ BSA.

Primary screening of chemical library

The primary screen was carried out using a library of 17 500 synthetic compounds from Laboratories for Chemical Biology Umeå (LCBU). The compounds, originating from ChemBridge Inc. (San Diego, CA, USA), were selected to cover a large chemical range, and they were generally limited to a mass below 600 Da. The compounds were stored in 384-well plates at 5 mM concentration in 100% dimethyl sulfoxide (DMSO) in appropriate conditions (darkness, dry and room temperature).

In a pilot study, the full compound library was screened against UGPase alone, with each compound tested at 10 μ M. This study failed to identify any compounds specifically affecting UGPase activity, but it provided a list of 65 compounds in the library that were having a strong effect on the assay system itself (i.e. targeting ATP-sulfurylase or luciferase). These false-positives were excluded in the next screening. Subsequently, both UGPase and USPase were separately screened against the full compound library, with each compound tested at 50 μ M (primary screen). When evaluating both target enzymes, six out of 112 (5.4%) of the examined plates failed to achieve a Z' score higher than 0.5 (production of these plates generally coincided with clogging of pipette tips) and were repeated. Accepted plates were analysed using the MScreen system (University of Michigan) (Jacob *et al.*, 2012). An active compound was defined as a substance that reduced UGPase or USPase activity by more than 70% and that had no effect on the assay in the pilot study. Compounds that passed these criteria were selected for cherry-picking and further validation (Figure S1b).

Secondary screening, dose-response and tertiary screen of chemical library

For secondary screening, effects of all cherry-picked compounds during primary screen were re-assayed (again using assay A). In parallel, the compounds were re-screened using assays containing neither UTP nor UGPase/USPase, and replacing the starting substrate (Glc-1-P) with PPi (2.5 μ M). Apart from the changes described above, the methodology and selection were similar to the primary screen. Compounds that again had significant effects on UGPase and/or USPase activities and had no or little effect on the assay system itself were selected. Fresh powder for each selected compound was acquired from LCBU, quality assessed, and used for further validation.

In a dose-response study, the effects of the selected compounds were tested at several concentrations (from 0.78 μ M to 50 μ M) against both UGPase and USPase using assay A, as described above. As a tertiary screen, the effect of compounds that showed a dose-dependent effect on UGPase or USPase were further validated by using two orthologous systems, in both the forward (UDP-Glc synthesis) and reverse (UDP-Glc degradation) direction of the target enzymes (assays B and C, respectively).

In vivo studies

Effects of selected inhibitors were tested *in vivo*, using two *A. thaliana* (Colombia ecotype) based systems – pollen germination test and growth of cell suspension culture. Pollen-based assays were carried out using pollen collected from several flowering plants (7–8-week-old) grown in a greenhouse under a 16 h light and 8 h dark regime at 22°C and 18°C, respectively. Pollen was germinated on glass slides or in liquid media using the optimal media and methods described by Boavida and McCormick (2007). Pollen germination tests were run in a growth room with temperature set at 22°C. As reported by Boavida and McCormick (2007), and confirmed here, this temperature was crucial for optimal pollen germination rates. Nevertheless, small temperature variations ($\pm 0.5^\circ\text{C}$) were inevitable, which may explain the differences in germination rate between the experiments – from ca. 25 to 60% (see Figures 3, 9 and 10). Germination rates and pollen tube lengths were scored using a Zeiss Axioplan 2 microscope, and the images were taken/processed with the publicly available ImageJ software.

For studies on effects of the inhibitors on growth of cell culture, we used a habituated stem-cell-like suspension culture from *A. thaliana* (Kunz *et al.*, 2014). The culture was maintained in conical flasks or in plates on an orbital shaker, in darkness, in growth medium containing 87 mM sucrose and full strength Murashige/Skoog nutrient solution with vitamins (Duchefa, Harleem, the Netherlands) at pH 5.7. Every 7 days, 5 ml of cells were subcultured in fresh media (final volume 50 ml), as described in Kunz *et al.* (2014). In order to miniaturize the system, cells were grown in 24-well plates (Sarstedt) and the final volume was 50% of the total well volume. After 7 days, cell viability was examined by fluorescein diacetate staining (400 cells were scored for three biological replicates per sample), as described by Pesquet and Tuominen (2011). The amount of cells was determined by comparing the optical density at 600 nm (OD_{600}) of the samples against the OD_{600} of a standard curve of known cell weights. The optical density was determined using a DU530 spectrophotometer (Beckman, USA).

To compare *in vivo* effects of SA and cmp #6D2, we used transgenic *Arabidopsis* plants transformed with a GUS construct under a SA-inducible promoter (pNPR1). Col-0:pNPR1::uidA seeds were ordered from The Nottingham *Arabidopsis* Stock Centre's homozygous collection (stock code: N6357) and grown as described above. Leaves were harvested from 21-day-old plants and treated with 5 mM SA or 5 μM #6D2 for 2 h at room temperature, followed by GUS staining, as described in Kunz *et al.* (2015).

Determination of QEPT for selected compounds

The QEPT value, which describes the likelihood for a given substance to cross root plasma membrane, was done for selected compounds, using the weightings and desirability functions described by Limmer and Burken (2014). The physicochemical descriptors (molecular weight, solubility, polar surface area, number of rotatable bonds and hydrogen-bond donors/acceptors) for each compound was downloaded from the ZINC 12 database (Irwin *et al.*, 2012).

Chemicals

All compounds identified during library screening and all cmp #6 analogs were from ChemBridge Inc., San Diego, CA, USA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Outcome and analyses of primary screen.

Figure S2. Dose-response of cmp #3, #6, #9, #10 and #11 against *Leishmania* USPase and barley UGPase (assay A).

Figure S3. Dose-response of cmp #6, #6D and #6D2 against barley UGPase (assay B).

Figure S4. Kinetics of barley UGPase with cmp #6D. Dixon plots are shown.

Figure S5. Effects of cmp #6D and #6D2 on activities of UGPase, Glc-6-P dehydrogenase (G6PDH), hexokinase (HXK) and invertase (INV).

Figure S6. Effects of cmp #6A, #6C, #6E, #6K and #6D1 as well as UDP-Glc on *Arabidopsis* pollen germination

Figure S7. Effects of *p*-coumaric acid (PA) and salicylic acid (SA) on barley UGPase activity and their *in vivo* effects in comparison to cmp #6D2.

Figure S8. Schematic description of overexpression and purification of *Arabidopsis thaliana* USPase.

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Supplemental files: Decker et al.: “Identification and characterization of inhibitors of UDP-glucose and UDP-sugar pyrophosphorylases for in vivo studies”

Fig. S1. Outcome and analyses of primary screen. (A) Quality of each LCBU plate, using the Z' -score calculated based on controls (Zhang et al., 1999); the grey and black squares represent UGPase and USPase screen, respectively. (B) Graphical representation of selection principle, using compound plate #16 against UGPase as an example ($Z' = 0.83$). Compounds which inhibited the target enzyme more than 70% and were not a known inhibitor of the assay-system were selected for further validation. N indicates number of repeats per plate.

Fig. S2. Dose response of cmp #3, #6, #9, #10 and #11 against *Leishmania* USPase and barley UGPase (assay A). All compounds were assayed (assay A) from 780 nM to 50 μM at non-saturating substrate concentrations. Right panel shows chemical structures of the compounds used, together with their Chembridge ID (CID) or ZINC number: cmp #3 (CID 7328760), #6 (ZINC 5898067), #9 (CID 5906647), #10 (CID 7488857) and #11 (CID 5617105). Bars represent means ±SD, n = 3. RLU, relative luminescence unit.

Fig. S3. Dose response of cmp #6, #6D and #6D2 against barley UGPase (assay C). Assay C (reverse reaction) was used, where UDP-Glc and PPi were kept at 0.03 and 0.04 mM, respectively.

Fig. S4. Kinetics of barley UGPase with cmp #6D. Dixon plots are shown. The K_i' values were calculated according to Cornish-Bowden (1974).

Fig. S5. Effects of cmp #6D and #6D2 on activities of UGPase, Glc-6-P dehydrogenase (G6PDH), hexokinase (HXK) and invertase (INV). DMSO refers to control containing 0.05% DMSO (v/v). The inhibitors were tested at 50 μ M each. A star (*) indicates statistical significance according to Student's t-test ($p < 0.05$).

Activity of barley **UGPase** was assayed using assay B with UTP and G1P at 0.125 mM and 0.165 mM, respectively, in the absence or presence of 50 μ M #6D or #6D2. Assays for yeast **G6PDH** (at 50 μ l total volume) contained 100 mM Hepes-NaOH (pH 7.5), 6.5 mM $MgCl_2$, 0.83 mM NADP, 1 mM Glu-6-P, and were supplemented with inhibitor (50 μ M) or water. Reactions were initiated with an aliquot of yeast G6PDH (Roche, Mannheim, Germany). Reaction mixture (total of 50 μ l) for yeast **HXK** contained 100 mM Hepes-NaOH (pH 7.5), 6.5 mM $MgCl_2$, 0.83 mM NADP, 2.7 mM ATP, 1 mM Glc, 1.5 U G6PDH, and was supplemented with inhibitor (50 μ M) or water. Reactions were initiated with an aliquot of yeast HXK (Roche, Mannheim, Germany). Reaction mixture (total of 50 μ l) for yeast **INV** contained 100 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 0.83 mM NADP, 2.7 mM ATP, 300 mM Suc, 1.5 U G6PDH, 1.5 U HXK, and was supplemented with inhibitor (50 μ M) or water. Reactions were initiated with an aliquot of yeast INV (β -fructosidase) (Roche, Mannheim, Germany).

Fig. S6. Effects of cmp #6A, #6C, #6E, #6K and #6D1 as well as UDP-Glc on *Arabidopsis* pollen germination. Control refers to 0.05% DMSO (v/v), whereas the inhibitors and UDP-Glc were supplemented at 5 μ M and 0.5 mM, respectively. Incubation was carried out for 4 h. Bars represent means $\pm$ SD of at least 2 biological repeats. A star (*) indicates statistical significance according to Student's t-test ($p < 0.05$).

Fig. S7. Effects of *p*-coumaric acid (PA) and salicylic acid (SA) on barley UGPase activity and their *in vivo* effects in comparison to cmp #6D2. (A) Structures of cmp #6D and #6D2 compared to those of PA and SA. For cmp #6D/#6D2, structural parts corresponding to those of PA and SA are shadowed in pink and green, respectively. (B) PA had no effect on UGPase activity nor on pollen germination. (C) SA had no effect on UGPase activity nor on pollen germination. SA induced GUS expression in *Arabidopsis* plants carrying an SA-inducible pNPR1::uidA construct, whereas cmp #6D2 had no effect. Bar = 100 μm.

Fig. S8. Schematic description of overexpression and purification of *Arabidopsis thaliana* USPase. The right panel represents DNA construct used for overexpression, and the left panel refers to a Coomassie Blue-stained gel following SDS-PAGE of the USPase at various stages of purification.

The pETtrx-USPase vector was prepared by cloning the full-length cDNA sequence of *AtUSPase* (*At5g52560*) between the *NcoI* and *NotI* sites on the vector, by ligating a *NcoI/XbaI* and a *XbaI/NotI* fragment (due to an internal *NcoI* site) with the digested vector. The sequenced (Eurofins MWG, Germany) vector was introduced to *E. coli* BL21(DE3) by electrotransformation. Cultures were grown in LB media for 4 h followed by induction with 1 mM isopropyl- β -thiogalactoside (IPTG) for 17 h in 10 °C. The harvested cells were lysed (in 20 mM Tris, pH 7.5, and 300 mM NaCl) by sonication and the soluble fraction (Sol) was isolated. This material was subsequently loaded onto a Co²⁺ TALON Resin (Clontech) column (equilibrated in 20 mM Tris, pH 7.8, and 300 mM NaCl) and the flow-through collected (Ft). The column was washed (20 mM Tris pH 7.0 and 300 mM NaCl) and subsequently the eluate (20 mM Tris, pH 7.0, 300 mM NaCl and 250 mM imidazole) was collected (E1). This eluate was desalted using a NAP-10 column (GE healthcare) and subsequently digested for 2 h in room-temperature by 100 μ g 6xHis-tagged TEV-protease (TEV). The material was loaded on a second Co²⁺ TALON Resin column and the flow-through fraction collected (E2). The enzyme was snap-frozen and stored at -80 °C in 20 mM Tris, pH 7.5, and 1 mM MgCl₂. M, protein markers with indicated molecular masses.