UDP-glucose pyrophosphorylase (UGPase) produces UDP-glucose which is essential for sucrose and polysaccharide synthesis. Using Arabidopsis, we demonstrated that two UGPase genes (UGP1 and UGP2) are differentially expressed in a variety of organs, with UGP1 being predominant. Co-expression analyses of UGP genes suggest that UGP1 is closely co-regulated with carbohydrate metabolism genes, late embryogenesis and seed loading, while UGP2 is co-regulated with stress response genes, fertilized flowers and photosynthetic genes. We have used Arabidopsis mutants for the UGP genes to characterize the role of both genes. The UGPase activity/protein was reduced by 70, 10 and 85% in ugp1, ugp2 and ugp1/ugp2 double mutant (DK) plants, respectively. A decrease in UGPase activity/protein was accompanied by an increase in expression of USP, a gene for UDP-sugar pyrophosphorylase, suggesting a compensatory mechanism. Generally, the mutants had no effects on soluble sugar/starch content (except in certain cases for DK plants), and there were no differences in cell wall composition/content between the wild type and the mutants. On the other hand, DK plants had greater hypocotyl and root lengths. When grown in the field, the mutants had as much as a 50% decrease in the number of seeds produced (consistent with a substantial decrease in field fitness), suggesting that they would be outcompeted in the field in a few generations. Overall, the data suggest that UGPase is not rate limiting for sucrose/starch and cell wall synthesis, but that it is essential in Arabidopsis.

Keywords: Callose • Cellulose • Cell wall synthesis • Seed fitness • Sucrose synthesis.

Abbreviations: ABRC, Arabidopsis Biological Resource Center; AIR, alcohol-insoluble residue; DK, double mutant; FBPase, fructose-1,6-bisphosphatase; GUS, β-glucuronidase; NASC, Nottingham Arabidopsis Stock Centre; ORF, open reading frame; RT–PCR, reverse transcription–PCR; SPS, sucrose phosphate synthase; TFA, trifluoroacetic acid; UGPase, UDP-glucose pyrophosphorylase; ugp, UGPase mutant; UGP, gene for UGPase; USPase, UDP-sugar pyrophosphorylase; USP, gene for USPase; UTR, untranslated region; wt, wild-type.

Introduction

UDP-glucose pyrophosphorylase (UGPase) (EC 2.7.7.9) is a ubiquitous enzyme found in plants, animals and bacteria, and produces UDP-glucose which is the major glycosyl donor for polysaccharides in all organisms (e.g. it is needed for sucrose synthesis, but also for cellulose and callose formation, and as a precursor to other nucleotide-sugars) (Kleczkowski 1994, Gibeaut 2000, Winter and Huber 2000, Johansson et al. 2002). The enzyme carries out a freely reversible reaction and thus can be involved in both anabolic and catabolic reactions in the cell, depending on the cellular metabolic status. The UGPase protein has been characterized from several plant species (Nakano et al. 1989, Elling, 1996, Meng et al. 2008), and a number of corresponding cDNA clones have been isolated (Katsube et al. 1991, Eimert et al. 1996, Pua et al. 2000, Pina and Errea 2008).

Whereas UGPase is probably not prone to significant regulation by metabolic effectors (Kleczkowski et al. 2004), other means of control have been postulated, including
oligomerization and post-translational modifications. For instance, barley UGPase has been found to oligomerize reversibly, which affects its activity and may represent a regulatory event (Martz et al. 2002, Geisler et al. 2004, McCoy et al. 2007). In yeast, UGPase is regulated by phosphorylation (Rutter et al. 2002), and in human and rice UGPase has been found to be subject to in vivo O- and N-glycosylation, respectively (Wells et al. 2003, Komatsu et al. 2009). UGPase protein was frequently detected as a major stress-responsive protein (Repetto et al. 2003, Carpentier et al. 2007, Zhu et al. 2007), most probably reflecting (post)transcriptional up-regulation of the UGP gene(s). For instance, UGP from Arabidopsis was strongly up-regulated by Pi deficiency conditions (Ciereszko et al. 2001a, Ciereszko et al. 2005) and cold stress, the latter acting via an ABA-insensitive pathway (Ciereszko et al. 2001b). The Arabidopsis UGP gene(s) was also up-regulated by sucrose, with the sucrose effect being transmitted via a hexokinase-independent pathway and independent from the Pi deficiency mechanism (Ciereszko et al. 2001b, Ciereszko et al. 2005). Changes in expression of UGP gene(s) were usually followed by changes, although modest, in UGPase activity/protein content.

Recently, it has become apparent that plants may contain isozymes of UGPase, encoded by different genes (Chen et al. 2007b, Meng et al. 2007, Meng et al. 2008). For instance, Arabidopsis contains two highly homologous UGPase genes, UGP1 (At3g03250) and UGP2 (At5g17310). The genes have a typical UGP gene organization, each having 18 exons and conserved intron–exon junctions. The genes code for proteins that contain all amino acid residues that were earlier reported as essential for UGPase catalysis and substrate binding (Katsube et al. 1991, Geisler et al. 2004, McCoy et al. 2007). Indeed, when cDNAs corresponding to each of the UGPase isozymes were overexpressed in Escherichia coli, the subsequently purified proteins displayed typical UGPase properties. Those characteristics included relatively high specific activities, high affinities for all substrates in both directions of the reaction, and specificity for UDP-glucose (pyrophosphorolytic direction) and glucose-1-P (synthesis direction) as substrates (Meng et al. 2008). Whereas both isozymes are predicted to be located in the cytosol, nothing is known about their in vivo roles in Arabidopsis, e.g. their relative involvement in sucrose synthesis or contribution to synthesis of cell wall polysaccharides.

To evaluate the role of UGP genes in Arabidopsis, we have investigated their expression (as well as UGPase activity/protein content) in a variety of tissues, and have analyzed their co-expression patterns against all Arabidopsis genes. Subsequently, we have identified both ugp1 and ugp2 T-DNA insertion mutants and prepared a ugp1/ugp2 double mutant (DK). For all the mutants [and wild-type (wt) plants] several parameters were studied, including UGPase activity/protein content, contents of soluble sugars and starch, cell wall content/composition, root/hypocotyl lengths as well as field fitness parameters. In short, the mutants were almost identical to the wt in most of the parameters studied, with, for example, only small or no changes found in carbohydrate status in the DK mutant, but the transgenics produced significantly lower number of seeds when compared with the wt. The results are discussed with respect to the role of UGPase in Arabidopsis, and put in a broader context of rate-limiting steps in carbohydrate synthesis and the role of UDP-glucose in plants.

Results and Discussion

UGP gene expression and UGPase activity/protein in Arabidopsis tissues/organs

Expression of both UGP genes as well as analyses of UGPase activity and its protein content were carried out in a variety of Arabidopsis tissues/organs (Fig. 1). Expression of UGP1 was, with the exception of flowers, higher than that of UGP2.
There was a relatively strong correlation between UGPase activity and UGPase protein content in all tissues/organs, but not between UGP gene expression and UGPase activity/protein. For instance, roots contained the highest UGPase activity/protein, but the overall expression of UGP genes was the lowest among the tissues/organs tested. From comparison with the positions of molecular mass marker proteins on SDS–PAGE, the estimated molecular mass for UGPases from Arabidopsis was about 51–53 kDa, which agrees with estimates for the proteins from potato and barley (Katsube et al. 1991, Eimert et al. 1996, Martz et al. 2002) and with calculated molecular masses (derived from cDNAs) of 51.6 and 51.7 kDa for UGPase1 and UGPase2, respectively (Meng et al. 2008).

**Sucrose-mediated differential expression of UGP1 and UGP2 genes**

We have previously demonstrated that, on Northern blots, UGP expression is up-regulated by sucrose in the leaves (Ciereszko et al. 2001b, Ciereszko et al. 2005). Sucrose effects on expression of the two UGP genes were confirmed both by quantitative PCR (data not shown) and using transgenic plants carrying UGP1- and UGP2-specific promoter–GUS (β-glucosidase) constructs (Fig. 2). Both UGP genes were up-regulated in cotyledons of sucrose-grown plants, but only UGP1 expression was evident for plants grown on medium without 5% sucrose. Also, based on GUS staining, both UGP genes were strongly expressed in anthers in flowers, and UGP1, but not UGP2, was expressed in seeds (Fig. 2). The latter finding was surprising, given that in an earlier study on isolated Arabidopsis embryos UGP2 expression was found greatly to exceed that of UGP1 (Baud and Graham 2006). A plausible explanation could be that what we observed was actually staining of the seed coat rather than whole seeds (including the embryo). If so, in our experiments it is the seed coat where the UGP1 expression appears to exceed that of UGP2.

Surprisingly, we could not detect any expression of UGP genes in roots of GUS plants (data not shown), and this is in contrast to our results with the use of quantitative PCR, where both UGP genes were found to be expressed, although to a lower extent than in other organs (Fig. 1). This may be due to the fact that regulatory motifs responsible for root expression are perhaps present further upstream than the promoters that were amplified for GUS studies, or that the relevant motifs that define expression in a given organ are in the 3’-untranslated region (UTR), as, for example, in the case of the potato APS gene of ADP-glucose pyrophosphorylase, a key enzyme of starch synthesis (Nakata and Okita 1996). Also, the promoter of UGP2, but not UGP1, contains a short nucleotide motif, CATGCATG, named UGPe-2, which was predicted to respond specifically to sucrose treatment (Geisler et al. 2006). The sucrose effect as seen in UGP2–GUS plants (Fig. 2) perhaps reflects the presence of this motif.

![Figure 2](https://example.com/figure2.png)  
**Fig. 2** Expression patterns of UGP1 and UGP2 genes in Arabidopsis plants expressing UGP–promoter–GUS constructs. (A–D) Cotyledons of agar-grown plantlets; (E–G) flowers (soil-grown plants); (H, I) young siliques (soil-grown plants); (J) mature siliques (soil-grown plants). For UGP2, there was little or no GUS staining in siliques, regardless of their developmental stage. The table at the bottom describes the relative expression of UGP1 and UGP2 genes (+, not expressed; ++, weakly expressed; +++ moderately expressed; ++++, strongly expressed). Suc, sucrose.
The UGPe-2 motif is not present in promoters of two sucrose-responsive UGP genes in aspen (Meng et al. 2007), but they contain another sucrose motif (SAKGCRCRG), called UGPe-1 (Geisler et al. 2006). Promoters of aspen UGP1 and UGP2 genes have one and four repeats of this motif, respectively.

**Co-expression analyses of UGP genes and USP with other genes**

Co-expression analyses were done for both UGP genes and for USP, a gene corresponding to so-called UGP-sugar pyrophosphorylase (USPase), which can non-specifically utilize UTP and glucose-1-P to produce UDP-glucose and PPi (Litterer et al. 2006). Both UGPase and USPase have little homology and are evolutionarily distinct (Geisler et al. 2004). Expression patterns of UGP1, UGP2 and USP (Fig. 3) were identified in a large collection of DNA microarrays including both abiotic stresses and differential tissue expression. All three genes were expressed above the genomic average in most tissues, with UGP1 strongly up-regulated in late embryogenesis and in seed loading, while UGP2 was up-regulated in roots and fertilized flowers, and suppressed in embryos and seeds. Whereas both UGP genes were sensitive to cold stress, UGP2 responded more quickly (after 3 h) and returned to pre-stress levels after 6 h, UGP1 responded to cold only after 12 h treatment, but remained high, and USP did not respond to cold. UGP2, but not UGP1, was induced strongly by heat shock, increased nitrate content and in seeds imbibed under white light (vs. dark imbibed). Both UGP genes were up-regulated in pollen, in high light in leaves, and responded to the circadian rhythm (Fig. 3).

For UGP1 and UGP2, the overall similarity of expression patterns in all microarray experiments tested was low (R = 0.18), on the border of being statistically significant, whereas there was no similarity at all between UGP genes and USP (R < 0.04). When all Arabidopsis genes were clustered on the basis of co-expression, the UGP and USP genes fell into separate clusters. UGP1 co-clustered with genes mainly involved in carbohydrate metabolism, especially starch metabolism (Fig. 3). On the other hand, genes with expression patterns most similar to UGP2 were involved in the light reactions of photosynthesis, including thioredoxin, plastocyanin, many heat shock proteins (not shown), zinc finger, WRKY and WD40-type transcription factors, as well as iso flavone reductase (Fig. 3). The fact that both UGP genes belong to two distinctive groups of co-regulated genes suggests that their expression is regulated by different mechanisms. USP was sorted to a very large cluster of genes with no real pattern of biological function.

**Impact of ugp mutants on UGPase activity/protein**

In the resources of the Arabidopsis Biological Resource Center (ABRC), we have identified putative Arabidopsis T-DNA mutants for UGP1 and UGP2 genes. The ugp1 mutant had a T-DNA insert in the 3′-UTR, whereas that of ugp2 had a T-DNA insertion in intron 16. Using semi-quantitative reverse transcription–PCR (RT–PCR), we found that the ugp1 mutant was ‘leaky’, still producing some UGP1 transcript, which included the full-length open reading frame (ORF). On the other hand, ugp2 most probably represented a complete knockout, because its UGP2 transcript lacked a substantial part at the 3′ end corresponding to 100 amino acids of the C-terminus of the derived protein (data not shown). We have identified homozygous ugp1 and ugp2 lines (see Materials and Methods) and, subsequently, have crossed ugp1 and ugp2 plants to obtain ugp1/ugp2 DK plants.

UGPase activities in the leaves of ugp1, ugp2 and DK plants were approximately 30, 90 and 15%, respectively, when compared with wt plants. For roots, the respective values were 30, 95 and 25% (Fig. 4). The lower UGPase activity in ugp mutants was accompanied by a decrease in UGPase protein, and generally there was a tight correlation between those two parameters. A tight correlation between UGPase activity and its protein was observed earlier for UGPases from Arabidopsis and *Populus* (aspen) plants (Ciereszko et al. 2001a, Ciereszko et al. 2001b, Ciereszko et al. 2005, Meng et al. 2007).

In the DK, the UGPase activity should, at least theoretically, correspond entirely to UGPase1, since UGP2 expression was entirely knocked out, whereas the ugp1 mutant was ‘leaky’ and the gene was expressed. There was, however, a possibility that a reduction in UGPase activity/protein in the mutants may be compensated by USPase, which can non-specifically carry out the UGPase reaction (Litterer et al. 2006). We have tested this by studying USP expression both in wt plants and in the DK background. All DK tissues contained at least four times lower UGPase activity than in corresponding tissues of wt plants (Fig. 1 vs. Fig. 5). In the DK, the expression of USP was increased in most tissues, especially in flowers and roots (Fig. 5). Interestingly, in those two organs, especially flowers, the activity of UGPase was less tightly correlated with UGPase protein, suggesting that part of the activity may indeed come from USPase rather than UGPase. The up-regulation of USP in those tissues (Fig. 5) can probably be regarded as a compensatory response, as frequently observed when one gene of a functionally related gene family is specifically blocked (e.g. Ruban et al. 2003).

**UGPase deficiency affects hypocotyl and root length**

The ugp1, ugp2 and DK transgenic plants were indistinguishable from the wt in terms of seed germination rate, growth rate, biomass production and flowering time (data not shown). However, for very young agar-grown plantlets, we observed differences in the length of roots and hypocotyls between DK mutants and wt plants. In those experiments,
we grew the plantlets in either light or dark conditions on agar media in the presence or absence of sucrose (Fig. 6). Generally, for dark-grown plants, the increased concentration of sucrose inhibited growth of hypocotyls. On the other hand, the roots were longer in sucrose-containing media (1 and 3%) than in those lacking sucrose. This was observed both for the wt and the DK plants, and was consistent with earlier studies showing that sucrose-grown plants have shorter hypocotyls and longer roots (e.g. Lee-Ho et al. 2007). In the dark, the DK mutant consistently had longer
hypocotyls and roots than the wt, irrespective of whether the plantlets were grown in the presence or absence of sucrose (Fig. 6). In the light, both wt and DK plantlets had very short hypocotyls, but the DK plants grown on sucrose still had significantly longer roots than the wt (Fig. 6).

The DK phenotype was similar to that of wt plants grown under mineral deficiency, e.g. Pi or nitrate deprivation. Those plants, at least at the young stage, frequently have longer roots when compared with plants grown under optimal conditions (Zhang and Forde 1998, Ciereszko et al. 2002, Karthikeyan et al. 2007). Interestingly, under Pi deficiency, UGPase content and activity markedly increased (Ciereszko et al. 2001a, Ciereszko et al. 2005), most probably to replenish internal Pi (by producing PPI which is then hydrolyzed to Pi by a pyrophosphatase). Plants with a decreased UGPase activity, as in DK, would obviously be less effective in terms of Pi recovery. However, the exact reason for differences in growth rates for wt and DK plantlets is unknown.

Is UGPase rate limiting in Arabidopsis?

In order to evaluate the impact of the decreased UGPase activity on carbohydrate status of the plants, we analyzed contents of soluble sugars and starch in the leaves. We have tested several growth conditions, including plantlets grown on agar plates and soil-grown plants. In all cases, the contents of soluble sugars and starch were similar for the wt and single mutants, and the only differences were found for soil-grown DK plants. In leaves of those plants, both starch and soluble sugars were slightly lower than in the wt (Fig. 7). This was more pronounced for leaves collected during the middle of the photoperiod when compared with those from the end of the photoperiod. On the other hand, young agar-grown DK plantlets were indistinguishable from the wt in terms of contents of starch and soluble sugars, with the possible exception of sucrose content, which was slightly lower in the DK than in the wt (Fig. 7).

Our data concerning Arabidopsis UGPase resemble those obtained by Zrenner et al. (1993) for UGPase from potato tubers where UGP expression was inhibited via ‘antisense’ interference. In those studies, no changes in carbohydrate contents were observed even in a transgenic line that had 96% inhibition of UGPase activity in the tuber. The authors concluded that UGPase exists in excess and that 4% of enzyme activity is sufficient for unperturbed metabolism. However, in another study of ‘antisense’-inhibited UGP in potatoes, the maximum inhibition of UGPase activity that was achieved was 50%, but this led to a considerable decrease in soluble sugar content (Spychalla et al. 1994, Borovkov et al. 1996). This suggested that UGPase constitutes a rate-limiting step in sucrose/glucose metabolism. These apparently fundamental differences in studies on the role of potato UGPase could perhaps be reconciled by differences in the methods/approaches that were used. For example, the ‘antisense genes’ differed in length and origin and were driven by different promoters. It has been shown that different lengths of the ‘antisense’ gene can affect the effectiveness of the ‘antisense’ attempts (Kuipers et al. 1995). Secondly, the tubers used were of different developmental stages: developing tubers...
(Zrenner et al. 1993) and stored tubers (Spychalla et al. 1994, Borovkov et al. 1996). At those stages, UGPase has different roles, reflecting its involvement in sucrose breakdown (developing tubers) and sucrose synthesis (stored tubers).

Thirdly, the possibility that potato may have distinct genes for UGPase which were differentially inhibited in those experiments cannot be excluded.

Regardless of the nature of discrepancies concerned with ‘antisense’ studies on the role of potato UGPase, our studies using defined Arabidopsis mutants of UGP genes have advantages and yield less ambiguous results because the effect can be ascribed to a specific gene, and not to a homologous gene family. Also, we have studied different tissues, in contrast to the work on potato where tubers were the focal point. Tubers are highly specialized for starch storage, and their sugar metabolism is not necessarily analogous to sugar metabolism in other parts of the plant, e.g. in leaves. However, as in any study involving mutants, the possibility cannot be excluded that a mutant (or ‘antisense’ approach) may bring about a compensatory effect. Such an effect in this study probably involves an increase in USP expression in most tissues of the DK plants (Fig. 5), which may potentially lead to an increased UGPase-like activity.

UGPase is located centrally in a pathway to sucrose synthesis in all plants. Thus, earlier studies on the possible rate-limiting role of enzymes in the same pathway could be of relevance. Those enzymes include sucrose phosphate synthase (SPS) and fructose bisphosphatase (FBPase), which are frequently considered as rate-limiting enzymes in sucrose synthesis (Edwards and Walker 1983, Winter and Huber 2000). A decrease in sucrose and starch contents, although small, as observed in our study (Fig. 7), is reminiscent of studies on transgenic Arabidopsis with a decreased expression of cytosolic SPS (Strand et al. 2000). In those studies, however, the effects were much stronger and the overall growth of plants was reduced by up to 50%, whereas in our ugp mutants the growth was similar to that of the wt. With respect to ‘antisense’ inhibition of cytosolic FBPase activity in potato tubers (Zrenner et al. 1996) and Arabidopsis (Strand et al. 2000), there was a marked increase in starch content, rather than a decrease (with growth reduction in Arabidopsis, but not in potato tubers). Thus, in terms of

![Fig. 6](image-url) The lengths of hypocotyls and roots in the wt and ugp1/ugp2 double-mutant (DK) of Arabidopsis. In (A), the plantlets were grown in darkness for 4 d. In (B), the plantlets were grown in the light (150 µmol m⁻² s⁻¹) for 5 d. For every experiment, about 150 plantlets were analyzed. Experiments were done in three replicates. The asterisk indicates a statistically significant difference. Hypo, hypocotyl; suc, sucrose.
effects on carbohydrate content, UGPase deficiency resembles more deficiency of SPS than of FBPase. Both SPS and UGPase are positioned downstream from the influx of starch-derived glucose (occurring in the night) into the sucrose pathway, in contrast to FBPase (Strand et al. 2000).

Effects of UGPase deficiency on cell wall contents/composition

Both cellulose synthase and callose synthase are located in plasmalemma and they both require UDP-glucose as a substrate. It has been assumed that sucrose synthase is the sole activity providing UDP-glucose for polysaccharide formation at the plasmalemma (Doblin et al. 2002); however, there is also evidence that UGPase may be involved. For instance, in tobacco, the overexpression of a UGP gene, although not increasing the cellulose content in terms of percent, was effective in increasing the total biomass and thus the overall cellulose yield from a given plant (Coleman et al. 2006). In Populus, a UGP gene is coordinately up-regulated with cellulose synthase during late cell expansion and secondary cell wall formation (Hertzberg et al. 2001), and the overexpression of bacterial UGPase in this species did increase cellulose (as well as soluble sugars and starch) content, even though growth of those plants was impaired (Coleman et al. 2007).

We have addressed the role of UGPase in cell wall formation in Arabidopsis by analyzing cell wall composition and contents in ugp mutants vs. the wt. The sugars analyzed included glucose, which is derived mainly, but not exclusively, from cellulose, the predominant carbohydrate in the cell walls, and its amount or its ratio to other wall components could have been affected by the mutants of UGP1 and UGP2. Three distinct tissues were used: young rosette leaves, mature rosette leaves and stems. First of all, quantitative analyses of the cell wall components were performed and no differences between the wt and mutants were observed (results not shown). Secondly, qualitative analyses of the cell wall composition showed no differences between the wt and ugp mutants (Fig. 8), with the composition in the three tissues analyzed in good agreement with previously published cell wall analyses of either seedlings (Mouille et al. 2006), leaves (Zablackis et al. 1995) or stems (Zhong et al. 2005). The observed differences in cell wall composition between the three tissues, with cellulose and pectin as major polymers in young plants and leaves and with cellulose and xylan prevalent in stems, were also as expected. When the results, shown here as mol%, were calculated as μg of sugar per μg of alcohol-insoluble residue (AIR) for both the wt and ugp mutants, again no differences in cell wall composition were observed.

The results do not exclude the possibility that in other tissues/organs or at different growth/developmental stages UGPase is rate limiting for cell wall formation. One example

FIG. 7 Contents of soluble sugars and starch in ugp1/ugp2 double mutant (DK) plants. The Arabidopsis plants were soil-grown (7-week-old plants) under conditions of an 8 h/16 h photoperiod and light intensity of 150 μmol m⁻² s⁻¹ (A, B) or grown on agar plates (2- to 3-week-old plants) under conditions of an 8 h/16 h photoperiod and light intensity of 50 μmol m⁻² s⁻¹ (C). In (A) and (B), mature leaves of the rosettes were collected during the middle of the photoperiod or at the end of photoperiod, respectively. In (C), whole seedlings were collected at midday. Please note that in (A) and (B) glucose contents were on the border of detection, and were not included into the graph. The asterisk indicates a statistically significant difference. Experiments were done in five biological replicates. Glc, glucose; Fru, fructose; Suc, sucrose.
The Is UGPase essential in Arabidopsis?

Concerning ORFs, Arabidopsis UGPases differ by only 32 amino acids, and the differences occur on the surface of the molecular masses and similar pI values (Meng et al. 2008).

When considering evolution of homologous genes, divergence may occur via mutations in the ORFs (affecting properties/regulation of gene-encoded proteins) or in the UTRs (affecting gene expression patterns) (Taylor and Raes 2004). Concerning ORFs, Arabidopsis UGPases differ by only 32 amino acids, and the differences occur on the surface of the molecules. (Meng et al. 2008).

Roles of UGPs in Arabidopsis

An obvious difficulty when studying the in vivo role of Arabidopsis UGPa in pollen formation is that they share very similar molecular masses and similar pl values (Meng et al. 2008). A similar situation occurs for two UGPa from aspen which share 94% identity at the protein level (Meng et al. 2007). When considering evolution of homologous genes, divergence may occur via mutations in the ORFs (affecting properties/regulation of gene-encoded proteins) or in the UTRs (affecting gene expression patterns) (Taylor and Raes 2004). Concerning ORFs, Arabidopsis UGPa differ by only 32 amino acids, and the differences occur on the surface of the molecule. (Meng et al. 2008).
protein rather than in the interior, and away from the active center (Meng et al. 2008). This may underlie evolutionary pressure for divergence of regulatory mechanisms for both proteins, e.g. concerning post-translational modification(s) or interactions with other proteins rather than metabolite regulation at the active site. For instance, UGPase isozymes in Arabidopsis have several amino acid motifs characteristic of protein kinase interaction sites and/or phosphorylation sites. Most of those motifs are shared by both isozymes, but there are some that are unique for each isozyme (Meng et al. 2008). Yeast UGPase was already shown to undergo reversible phosphorylation, with the phosphorylation event controlling partition of carbon to the glycogen and cell walls (Rutter et al. 2002). Other possibilities of regulation, earlier shown for plant UGPases, include in vivo binding of 14:3:3 proteins (Alexander and Morris, 2006), glycosylation (Wells et al. 2003, Komatsu et al. 2009) and acetylation (Chen et al. 2007a). Also, barley UGPase was demonstrated to be able to undergo oligomerization, with the deoligomerization step shown to be rate limiting (Martz et al. 2002). Crystal structure analyses of Arabidopsis UGPase1 have revealed that the protein indeed exists as a mixture of monomers and dimers, the latter possibly representing an inactive form (McCoy et al. 2007).

A clue for the role of a given UGPase protein may come from analyses of expression of the corresponding UGP genes. Both UGP1 and UGP2 are differentially expressed in Arabidopsis tissues (Figs. 1, 2) and are co-regulated by different mechanisms (Fig. 3). For instance, in leaves and stems, UGP1 expression far exceeded that of UGP2 (Fig. 1), suggesting a specific role for the former in those organs. Differential regulation at the tissue/organ level, and in response to sucrose and environmental signals, was earlier observed for two highly homologous UGP genes in aspen (Meng et al. 2007). Based on this and on the overall expression of both UGP genes in different tissues/organs, aspen UGP1 was suggested to function as the major, housekeeping gene, whereas aspen UGP2 has probably more of a back-up function (Meng et al. 2007). In Arabidopsis, UGP1 also appears to be the major UGPase gene, predominantly expressed in most tissues (Fig. 1) but also more sensitive to sucrose and cold regulation (data not shown, see also Fig. 3).

Based on co-expression analyses (Fig. 3), the UGP1 gene may deliver activity required for normal metabolic flux in most tissues, whereas the expression pattern of UGP2 is consistent with additional flux in situations where surges of supply (an increase in photosynthesis) or demand (stress) cannot be met through up-regulation of UGP1 alone. On the other hand, based on experimental data, the observed differences and/or changes in expression of UGP genes, frequently quite dramatic (Fig. 1, see also Meng et al. 2007), were usually accompanied by only modest changes in overall UGPase activity/protein and it was impossible, based on that, to identify relative changes between UGPase isozymes. More
data directly linking a specific UGP gene with a specific UGPase protein would be needed for proper evaluation.

## Materials and Methods

### Plant material and growth conditions

Seeds of wt and T-DNA insertion mutants ugp1 and ugp2 of Arabidopsis thaliana (L.) Heynh., ecotype Col-0, were acquired from the ABRC of the Ohio State University, USA. The stock numbers for the ugp1 and ugp2 seeds were N559624 and N515899, respectively. Homozygous lines were identified by RT–PCR on total RNA from individual plants, using gene-specific primers (Table 1). Those individuals whose target region could not be amplified were picked out as homozygote lines. The DKs were obtained by crossing ugp1 and ugp2, the resulting plants were self-pollinated and then the double homozygous were screened for by three-primer PCR that contained two gene-specific primers and one primer corresponding to the sequence of the T-DNA left border, using the genomic DNA as template. The primers used are listed in Table 1.

Generally, the wt and mutant plants were soil-grown in controlled climate growth chambers with a photoperiod of 8 h/16 h with 130–150 µmol m⁻² s⁻¹ and a day/night temperature of 23/18 °C. Similar conditions were employed for plantlets grown on agar plates, using Murashige–Skoog medium (routinely supplemented with 1% sucrose, unless otherwise stated). For cell wall component analyses and hypocotyl/root length measurements, plants or plantlets were grown under a 16 h/8 h photoperiod, with the same light intensity and temperature as above.

### RNA and DNA methods

For gene expression analyses, total RNA from Arabidopsis tissues was extracted by using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). cDNA synthesis was run with 0.3 µg of total RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative PCRs and data processing were done as described by Meng et al. (2007). Nucleotide primers used are described in Table 1. DNA sequencing was done by using a BigDye Cycle sequencing kit (Perkin Elmer) on an ABI 377 sequencing machine (Perkin Elmer).

### GUS constructs and GUS staining

The promoter regions of UGP1 and UGP2 genes (approximately 1,300 and 2,500 bp immediately upstream of the ATG initiation codon, respectively) were amplified from Arabidopsis genomic DNA with turbo pfu polymerase (Stratagene, La Jolla, USA), and then cloned into the multi-cloning site of pCV812 which includes the uidA (GUS) coding region (Koncz et al. 1989). Nucleotide primers used are listed in Table 1. GUS activity was detected following the protocol of Geisler et al. (2002).

### Soluble sugar/starch determination

Plant material was collected at given time points and immediately frozen in liquid nitrogen. Soluble sugar and starch were extracted and quantified by enzymatic methods as described by Ciereszko et al. (2001a)

### Cell wall monosaccharide analyses

AIR was prepared and destarched using thermostable α-amylase (Megazyme, Wicklow, Ireland) followed by amyloglucosidase (Megazyme, Wicklow, Ireland) as described by Fry (1988) and Harholt et al. (2006). The pellet (AIR) was dried under vacuum and weighed. Samples were hydrolyzed in 2 M trifluoroacetic acid (TFA) for 1 h at 120 °C. TFA was removed by drying under vacuum overnight. In order to measure the amount of crystalline cellulose, AIR was solubilized in 72% sulfuric acid for 1 h at room temperature. Water was added to a final concentration of sulfuric acid of 6.4%. The samples

---

**Table 1** List of DNA primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PromUGP1_forw</td>
<td>GCTCGAGGATCCATGACCTTTT</td>
</tr>
<tr>
<td>PromUGP1_rev</td>
<td>CAGATGACGTTTCCTGTATAG</td>
</tr>
<tr>
<td>PromUGP2_forw</td>
<td>GTGCGTCTTCTTCTTCTTCTCT</td>
</tr>
<tr>
<td>PromUGP2_rev</td>
<td>ATCTTTTCTTCTTCTCTTCTTCT</td>
</tr>
<tr>
<td>RTPCR_ugp1_forw</td>
<td>GGACATGACGTTTCCTGTATAG</td>
</tr>
<tr>
<td>RTPCR_ugp1_rev</td>
<td>GTTCTGTTCCCTGCAGAAAATG</td>
</tr>
<tr>
<td>RTPCR_ugp2_forw</td>
<td>TCAGTTCACCATCAGTCATCAG</td>
</tr>
<tr>
<td>RTPCR_ugp2_rev</td>
<td>CAGTTCACCATCAGTCATCAG</td>
</tr>
</tbody>
</table>

The primers were used to amplify UGP promoters, to screen for homozygous ugp mutations, to determine transcript abundance for both UGP genes and USP, and to amplify 18S RNA. Prom, promoter; forw, forward primer; rev, reverse primer; hmz, primer used for homozygote screening; RTPCR, reverse transcription–PCR; qPCR, quantitative PCR.

---

qPCR_UGP1_forw CAACTTTTCTTCTTCTTCTTCTCT
qPCR_UGP1_rev AGGGAGATTCTCAGTGGTGGC
qPCR_UGP2_forw TCCGCTTCTTCTTCTTCTTCTTCT
qPCR_UGP2_rev CATCCGCTAAGAAAGAGTTCA
qPCR_USP_forw TCACATGGAACAAAAATG
qPCR_USP_rev ACACCTCTCAAGGTCACACC
qPCR_18S_forw TCAACCTTCGATGTTAGATGAGT
qPCR_18S_rev CCGGTCAAGGTAGGTTATTT

The primers were used to amplify 18S rRNA. Prom, promoter; forw, forward primer; rev, reverse primer; USP, primer for the left border of T-DNA insertion; UGP, primer for the UDP-sugar pyrophosphorylase gene (identical to those used by Kotake et al. 2007).
were then hydrolyzed for 1 h at 120°C followed by neutralization and removal of sulfate using BaOH and barium carbonate as described by Fry (1988). The monosaccharide composition was subsequently determined on the TFA- and H₂SO₄-hydrolyzed samples by HPAEC-PAD detection using a PA20 column ( Dionex, Sunnyvale, CA, USA), as described previously (Øbro et al. 2004). Monosaccharide standards were from Sigma-Aldrich and included l-fucose, l-rhamnose, l-arabinose, d-galactose, d-glucose, d-xylene, d-galacturonate and d-glucuronate. For verification of the response factors, a standard calibration was performed before analyses of each batch of samples.

**UGPase activity and immunoblotting**

The protein extraction and enzymatic activity of UGPase were performed essentially as in Meng et al. (2007). Assays, started by addition of PPI, were carried out by coupling glucose-1-P production to formation of NADPH at 340 nm using a Beckman DU 530 spectrophotometer. One unit of UGPase activity was defined as 1 µmol NADPH formed per min.

Following Western blot (after SDS–PAGE), the UGPase protein was detected with rabbit antibodies (10,000-fold diluted) raised against barley UGPase, followed by donkey anti-rabbit IgG coupled to peroxidase (Amersham, Upplsa, Sweden). The labeling was detected with the ECL kit (Amersham, Sweden). Rabbit antibodies against purified heterologously expressed barley UGPase (Martz et al. 2002) were produced by AgriSera AB (Vännäs, Sweden).

**Field fitness experiments**

Both wt and UGPase-deficient plants were grown outdoors from July to August 2006 in a garden close to the Umeå Plant Science Center in Umeå, Sweden. To provide the plants with conditions resembling as closely as possible those of a natural population, they were not watered, fertilized or treated with pesticides. The plants were grown in parallel with other Arabidopsis transgenics; characterization of those plants has already been reported (Frenkel et al. 2007).

**Co-expression analyses**

Transcriptomic data sets from publically available NASC (Nottingham Arabidopsis Stock Centre; http://arabidopsis.info) and GEO (http://www.ncbi.nlm.nih.gov/geo/) databases were assembled into a single database of 1,301 experimental conditions, tissue types and mutants. Microarrays were selected for different tissues and stresses, including treatment with sucrose (100 mM), cold (4°C), heat (38°C), all hormones (at 10 and/or 100 µM) and dissection of individual tissues. For the upper panel of Fig. 3, tissues included roots (right, soil grown; left, agar grown), leaves (by stage; right to left is cotyledon to juvenile to cauline leaves), stems (right, basal; left, apical), flowers (by stage; right, early, left, late; also by organ sepal, petal, stamen, carpel), embryos and developing seeds from early (right-most) to late (left-most) stages. The last seed column is for dry seeds. Experiments for abiotic stress (lower panel in Fig. 3) taken from NASC Affywatch represent time courses (0.5, 1, 3, 6, 12 and 24 h) following stress application. Tissues sampled were shoots (left columns) and roots (right columns). Drought = 15 min dry air stream until 10% loss of fresh weight (Schmid et al. 2005); salt = transfer to plates containing 150 mM NaCl; cold = transfer to 4°C; heat = transfer to 38°C for 3 h, then recovery at 22°C; mannitol = transfer to 300 mM mannitol; wounding = leaves punctured with pins; sugars = sucrose 100 mM applied in light (left) and darkness (right); hormones (right to left) applied at 10 µM and sampled at 0.5, 1 and 3 h, were salicylic acid, jasmonic acid, ABA, indole acetic acid, gibberellin applied to seeds, zeatin, brassinolide.

The data from microarrays were subjected to K-means centroid clustering using Cluster 3.0 software (Eisen et al. 2008) which groups genes into 500 clusters on the basis of a similar pattern of expression (as determined by the Pearson correlation) across the stress/hormone/sugar data sets, or separately across the tissues data set. Heatmaps were generated in MS-Excel.

**Funding**

The Swedish Research Council.

**References**


(Received February 25, 2009; Accepted April 7, 2009)