1 **Trehalose 6-phosphate regulates photosynthesis and assimilate partitioning in**
2 **reproductive tissue**

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14 **Short title:** T6P regulates maize resource allocation

15 **One sentence summary:** Decreased T6P in phloem cells of maize female
16 reproductive tissue causes opposing changes in primary and secondary metabolism
17 and a shift in assimilates within cobs in favour of florets, simultaneously increasing
18 photosynthetic rate.

19 **Author contributions:** SSB, MLN and MJP directed and designed the research.
20 MO, LFP and CAG performed experiments. JC analyzed data. JC, MLN, SSB and
21 MJP wrote the paper, which all authors edited and approved.

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ABSTRACT

Transgenic maize (*Zea mays*) that expresses rice (*Oryza sativa*) *TREHALOSE PHOSPHATE PHOSPHATASE1* (*TPP1*) from the rice *MADS6* promoter, which is active over the flowering period, produces higher yields than wild type. This yield increase occurs with or without drought conditions during flowering. To understand the mechanistic basis of the increased yield, we characterised gene expression and metabolite profiles in leaves and developing female reproductive tissue – comprising florets, node, pith and shank – over the flowering period with and without drought. The *MADS6* promoter was most active in the vasculature, particularly phloem companion cells in florets and pith, consistent with the largest decreases in trehalose 6-phosphate (T6P) levels (two- to threefold) being found in pith and florets. Low T6P led to decreased gene expression for primary metabolism and increased gene expression for secondary metabolism, particularly lipid-related pathways. Despite similar changes in gene expression, the pith and floret displayed opposing assimilate profiles: sugars, sugar phosphates, amino acids and lipids increased in florets, but decreased in pith. Possibly explaining this assimilate distribution, seven *SWEET* genes were found to be upregulated in the transgenic plants. SnRK1 activity and the, expression of the gene for the SnRK1 beta subunit, expression of SnRK1 marker genes, and endogenous trehalose pathway genes were also altered. Furthermore, leaves of the transgenic maize maintained a higher photosynthetic rate for a longer period compared to wild type. In conclusion, we found that decreasing T6P in reproductive tissues downregulates primary metabolism and upregulates secondary metabolism, resulting in different metabolite profiles in component tissues. Our data implicate T6P/ SnRK1 as a major regulator of whole-plant resource allocation for crop yield improvement.

Key words: Trehalose 6-phosphate, maize, SnRK1, sucrose, photosynthesis, crop yields, drought

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INTRODUCTION

To avoid future food shortfalls, crop yields need to increase by more than is achievable at present by current crop improvement methods (Ray et al., 2013). It is also necessary to develop crops that are more stable in the face of increased climatic variability (Boyer et al., 2013). Hence, productivity combined with resilience is a sought-after goal. Improving crop performance under drought is complex because the effects of water availability on crop yield depend on crop developmental stage and genetic factors. The flowering period is particularly sensitive to drought (Boyer and Westgate, 2004); restriction of water at this time can decrease seed set, final seed number and harvested seed yield (Schussler et al., 1991a, b). Kernel abortion during drought at flowering can be alleviated by supplying sucrose to reproductive tissue (Zinselmeier, 1995a, b). Consequently, sucrose metabolism in reproductive tissue has been proposed as a target to alleviate the effects of drought during the reproductive period (Boyer and McLaughlin, 2007). Rather than directly targeting sucrose metabolism, it has been proposed that regulating the metabolism and utilisation of sucrose could be a more feasible target to alter assimilate partitioning (Boyer and McLaughlin, 2007).

The trehalose pathway is an important regulator of sucrose utilisation in plants (Schluepmann et al., 2003). Trehalose 6-phosphate (T6P), the precursor of trehalose, responds to sucrose likely as a signal of sucrose availability (Lunn et al., 2006; Martinez-Barajas et al., 2011; Nunes et al., 2013a; Yadav et al., 2014). Altering levels of T6P causes changes in gene expression (Nunes et al., 2013a), plant metabolism (Zhang et al., 2009; Figueroa et al., 2016) and growth (Nunes et al., 2013a), such that metabolic reprogramming occurs in light of sucrose availability. T6P can regulate starch levels through starch synthesis and breakdown (Kolbe et al., 2005; Martins et al., 2013) and enables the coordination of organic and amino acid metabolism with carbon availability (Figueroa et al., 2016). Such whole-scale effects are likely to be mediated by signal transduction and interaction with the feast/ famine protein kinase, SnRK1 (Zhang et al., 2009; Delatte et al., 2011; Nunes et al., 2013a, b; Tsai and Gazzarini, 2014). By mediating such effects on metabolism, growth and development, T6P ensures effective use of sucrose in addition to maintaining sucrose homeostasis. Yadav et al. (2014) have put forward a theory of the T6P: sucrose nexus; T6P levels could alter both the use and allocation of sucrose by increasing gene expression for the use of sucrose and mediating allocation by

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94 perturbing sucrose homeostasis. There have been many reports of associations
95 between the trehalose pathway and drought tolerance but no detailed mechanistic
96 basis for such a correlation. The abundance of trehalose itself is too low to provide
97 osmotic or oxidative stress protection against desiccation. Constitutive expression of
98 trehalose pathway transgenes to alter T6P accumulation has produced examples of
99 improved drought tolerance, but this may be because of reduced growth, which
100 decreases water loss and improves survival, and does not improve productivity as is
101 required in agriculture (Romero et al., 1997; Cortina and Cuilianez-Macia, 2005).

102 Nuccio et al. (2015) targeted changes in T6P abundance in reproductive
103 tissue during the flowering period using a rice (*Oryza sativa*) *MADS6*: trehalose
104 phosphate phosphatase (*OSMADS6: TPP1*) construct to alter sucrose metabolism
105 for yield preservation during drought. This modification simultaneously decreased
106 T6P and increased sucrose in female florets five days before pollination (Nuccio et
107 al., 2015). In extensive field trials over several years and locations, the transgenic
108 maize (*Zea mays*) demonstrated significantly improved yield, with and without
109 drought during the flowering period, through enhanced kernel set. This provides one
110 of very few reports wherein transgenic technology that has modified an intrinsic plant
111 process has substantially improved yield and was reproducible in the field
112 environment. Despite the importance of this success, little is known of the
113 mechanistic details that underpin this yield improvement.

114 In the current study, we performed a detailed analysis of plants that were
115 higher yielding than wild type in the field trials of Nuccio et al. (2015). We started
116 from existing models of the mode of action of T6P through SnRK1. Reproductive
117 tissue was sectioned into ear florets (female reproductive structures), pith (the
118 vascular core of the ear), node (the vasculature in the stalk where the ear emerges)
119 and shank (the branch where the ear attaches to the stalk). *OSMADS6: TPP1*
120 expression was found to be associated with phloem tissue in these structures and
121 transgene expression was greatest in florets and pith. Consistent with decreased
122 T6P concentrations, primary metabolic pathways were downregulated and
123 secondary metabolic pathways were upregulated in the tissues where *OSMADS6*
124 promoter drove GUS expression. This altered the distribution in the component
125 tissues of the reproductive structures away from pith toward the ear florets. *SWEET*
126 genes were the only class of gene associated with assimilate transfer that were
127 consistently affected in pith and florets. There were also changes in the expression

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128 of SnRK1 marker genes, endogenous trehalose pathway genes and the gene
129 encoding the SnRK1 beta subunit. Leaves of transgenic plants maintained higher
130 rates of photosynthesis for longer during the reproductive period. Our results provide
131 evidence that T6P/ SnRK1 acts as a central regulator of the balance between
132 primary and secondary metabolism, assimilate distribution and the whole-plant
133 source–sink interaction for crop yield improvement.

134

135 **RESULTS**

136 **Expression of *OSMADS6: TPP1* During the Flowering Period**

137 Female reproductive and leaf tissues were sampled for profiling analysis at 5-day
138 intervals beginning at silk emergence, 5 days before pollination (-5), under controlled
139 environmental conditions. Expression of *OSMADS6: TPP1* reached its highest levels
140 in pith tissue compared to node, shank and floret (Fig. 1A, B). There were different
141 *OSMADS6: TPP1* trends with respect to development in each tissue with expression
142 increasing over time in shank and pith but decreasing over time in florets. Activity
143 was most constant in the node during development. Analysis of *OSMADS6: GUS*
144 shows *OSMADS6* expression localised to vasculature in all tissues, and phloem
145 companion cells in particular (Fig. 2, Supplementary Fig. S1 and S2). Figure 1 shows
146 that *OSMADS6: TPP1* transcript was detected in leaves but, when leaf tissue from
147 *OSMADS6: GUS* plants was evaluated for GUS activity by histochemical analysis,
148 no evidence of enzyme activity was found (Supplementary Fig. S1). Hence,
149 *OSMADS6* does not appear to direct protein expression in leaves.

150

151 **T6P and Trehalose**

152 In wild-type reproductive tissue under well-watered (unstressed, US) conditions, T6P
153 abundance ranged from 4 nmol g^{-1} FW in the node, where it was most stable during
154 development compared to other reproductive tissues, to 60 nmol g^{-1} FW in the shank
155 5 days before pollination (-5) (Fig. 3A). T6P then fell to less than 10 nmol g^{-1} FW over
156 the next 15 days (0, 5, 10). Overall during development, T6P levels were highest in
157 florets and pith, between 32-60 nmol g^{-1} FW and 11-30 nmol g^{-1} FW, respectively.
158 T6P levels in leaves were around 5 nmol g^{-1} FW. In shank, the large peak of T6P 5
159 days before pollination was increased by drought to 102 nmol g^{-1} FW. Drought also
160 increased T6P in pith, but decreased it at later time points in florets. *OSMADS6:*
161 *TPP1* resulted in two- to threefold less T6P in pith and floret tissue under both well-

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162 watered and drought conditions. The transgene barely affected T6P levels in node

163 and leaves and increased T6P only twofold at the first time point in shank tissue.

164 Trehalose content followed the same pattern as T6P for tissue type and

165 developmental stage, with a 40 nmol g^{-1} FW peak of trehalose in shank tissue at day

166 -5 (Fig. 3C). Drought increased trehalose in node (Fig. 3D). *OSMADS6: TPP1* had

167 little effect on trehalose, increasing it slightly in node but decreasing it in pith and

168 florets. Trehalose was barely detectable in leaves.

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Metabolite Profiling

171 The largest effects of *OSMADS6: TPP1* on metabolite profiles were found in floret

172 and pith under well-watered and drought conditions (Fig. 4A, B, Supplementary Fig.

173 3A, B), which coincided with the largest expression of transgene and largest effect of

174 the transgene on T6P (Fig. 3A, B). The same trends were observed under well-

175 watered and drought conditions. The changes in metabolites in floret and pith were

176 largely in opposite directions, particularly for sucrose, glucose and fructose, and

177 amino acids, which were increased in florets but decreased in pith, although amino

178 acids in floret were unchanged or decreased at the last two time points. Levels of

179 xylose and xylulose were decreased in both tissues. Adenosine monophosphate

180 (AMP) increased in florets and decreased in pith in well-watered conditions.

181 Contents of phospholipids, sphingolipids and sterols were increased in florets

182 throughout development and were unchanged or decreased in pith. Node tissue was

183 similar to pith with regard to amino acids, but dissimilar to pith for sugars which

184 increased in node. Patterns of changes were less clear for shank and leaf, although

185 leaves of transgenic plants had more carbohydrates than wild type. Allantoin was the

186 only metabolite that increased in all tissues in transgenic plants. The known link

187 between T6P and sucrose (Nunes et al., 2013a; Yadav et al., 2014) was broken in

188 floret and node where a decrease in T6P (Fig. 3A, B) was related to more sucrose.

189 Increased sucrose in leaves did not correspond to more T6P. In pith and shank,

190 however, less T6P (Fig. 3A, B) was related to less sucrose (Supplementary Fig. 3A,

191 B).

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Gene Expression

194 Gene expression analysis from RNAseq analyses can be summarised by

195 categorizing genes significantly increased or decreased in both transgenic lines

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compared to wild type in all tissues under well-watered and drought conditions (Fig. 5, Supplementary Tables S1-4). The Roast algorithm was used to identify biochemical pathways significantly perturbed (Fig. 6). Overall, transgenic pith tissue exhibited the greatest changes in gene expression due to transgene followed by floret, shank, node and leaf. There were no large differences between well-watered and drought conditions in numbers or types of genes affected. A total of 67 pathway categories were perturbed by the transgene in both events at two or more time points in at least one tissue in either unstressed or drought stressed plants (Fig. 6). Overall, expression of primary metabolic pathways (major flux e.g. sugar nucleotides, starch, cell wall, amino acids) was decreased, particularly in pith and floret, whereas expression of genes for secondary metabolic pathways (minor flux e.g. lipids, trehalose) was increased (Fig. 6).

SnRK1 Marker Gene Expression

Given the known effects of SnRK1 on metabolic pathways (Zhang et al., 2009; Figueroa et al., 2016), we quantified the extent of changes in SnRK1 marker gene expression previously identified in Baena-Gonzalez et al. (2007), Zhang et al. (2009), and Martinez-Barajas et al. (2011). Maize orthologues of Arabidopsis (*Arabidopsis thaliana*) genes reported to be regulated by SnRK1 were established using a combination of OrthoMCL, reciprocal best BLAST match and gene synteny (Li et al., 2003). Putative maize orthologues (183 induced markers, 242 repressed markers) are reported in Supplementary Tables S5A and S5B. Of those that changed in both lines on at least one time point, 23 orthologues of SnRK1 markers normally induced by SnRK1 were induced under unstressed conditions (US) in pith and 25 were induced under drought conditions (DS) (Fig. 7A). Also in pith, 14 maize orthologues of Arabidopsis genes reported to be repressed by SnRK1 were repressed in both events in at least one time point tested under unstressed conditions (US) and 19 under drought conditions (DS) (Fig. 7B). Changes in SnRK1 markers were also observed in florets (Fig. 7A, Supplementary Table S5B).

SnRK1 Activity and Gene Expression

In unstressed plants, SnRK1 activities were higher in transgenic floret tissues compared to wild type at day 0 and day 10 (Fig. 8A, Supplementary Table S6). In pith, SnRK1 activity was higher in transgenics compared to wild type at day 10.

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SnRK1β expression increased in transgenics compared to wild type in pith and floret tissue whereas expression of the gene encoding the SnRK1 AKIN11 subunit decreased in pith (Fig. 8B). SnRK1 activity was inhibited by T6P in floret and pith tissues (Fig. 8A). Combined with decreases in T6P (Fig. 3), it is likely that *in vivo* activities of SnRK1 were significantly higher in transgenics compared to wild type.

Trehalose Pathway

The endogenous trehalose biosynthetic pathway was one of the few biosynthetic pathways to be upregulated in transgenics compared to wild type (Fig. 6). The gene set defined by Henry et al. (2014) was analysed in more detail. There were large changes in pith and, to a lesser extent, florets (Fig. 9, Supplementary Table S7) in well-watered (US) and drought-stressed tissues (DS). Genes encoding class II trehalose phosphate synthase (TPS) and TPPB were induced in transgenics compared to wild type in well-watered and drought conditions, whereas *TPPA* was repressed. *TPS1* was also induced in pith.

SWEET Genes

We next sought to explain changes in the distribution of assimilate between pith and floret. We found that *SWEET* genes were the only class of genes involved in transport or efflux that had significantly altered expression levels (Fig. 10). Seven *SWEET* genes were upregulated across the reproductive tissue, with greatest changes in pith (Fig. 10, Supplementary Table S8). No significant changes in expression were found for genes encoding sucrose transporters; amino acid transporter gene expression decreased, whereas expression of genes for two nitrate transporters increased in pith (Fig. 11).

Allantoinase

Given the consistent change in allantoin levels in most of the transgenic samples, expression of genes involved in its metabolism was examined. Allantoinase, which metabolises allantoin to allantoate, was decreased in all tissue without drought except florets, and in all tissues under drought conditions (Fig. 11). Changes were compared to other genes putatively involved in nitrogen metabolism, none of which changed as consistently as allantoinase.

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Photosynthesis

Rates of CO_2 uptake were determined in the leaf adjacent to the developing cob and the leaf above the developing ear under both well-watered and drought conditions. CO_2 uptake was up to 54% higher in transgenics compared to wild type (Fig. 12), with the largest percentage increase in primary leaf under drought (Fig. 12B). Effects were greatest at day 0 and day 5. There were no differences between transgenic and wild type in secondary leaves under drought (Fig. 12D). Analysis of gene expression in primary leaves showed upregulation of genes related to chloroplast and sucrose biosynthesis in unstressed tissue and decreases in chloroplast processes in drought-stressed leaves (Fig. 13).

DISCUSSION

Expression of a trehalose pathway gene linked to the rice *MADS6* promoter (*OSMADS6: TPP1*) in maize reproductive tissue was previously shown to increase maize yield in the field both with and without drought during the 2- to 3-week flowering period (Nuccio et al., 2015). This is one of the very few reports wherein genetically modifying an intrinsic process increases yield in field conditions. It is important to determine the biochemical and molecular basis of the yield increase to inform and develop crop improvement strategies. Four main conclusions can be drawn from this study. Firstly, localisation of *OSMADS6: TPP1* transgene expression in phloem cells of pith and florets lowered T6P levels two- to threefold. This resulted in the downregulation of primary metabolic pathways and upregulation of secondary metabolism, particularly lipid-related pathways, most strongly in pith. Secondly, this *OSMADS6: TPP1* transgene expression resulted in opposing changes in metabolites: increased sugars, amino acids and lipids in florets but decreased levels in pith; altered *SWEET* gene expression could account for assimilate transfer from pith towards floret. Allantoin was the only metabolite to increase in all tissues associated with the downregulation of allantoinase transcript in transgenic lines. Thirdly, a higher leaf photosynthetic rate in transgenics was likely to be in response to the enhanced movement of assimilate to florets. Fourthly, changes in SnRK1 activity and in the expression of its subunit genes as well of SnRK1 marker genes and endogenous trehalose pathway genes implicate T6P/ SnRK1 as a central mechanism of assimilate partitioning, allocation and source-sink regulation in crops.

Decreasing T6P in reproductive tissue downregulates primary metabolism and upregulates secondary metabolism, but causes distinct changes in metabolite profiles in component tissues

Following the current thinking on the role of T6P in plants, a decrease in T6P would be expected to repress the biosynthetic activity of metabolic pathways (Zhang et al., 2009; Figueroa et al., 2016). This is observed in the reproductive tissues in this study with regard to primary metabolism (sugar nucleotide, amino acids, starch and cell wall, Fig. 6). However, an increase in the activity of the pathways of lipid biosynthesis was observed. This represents a refinement of the earlier hypothesis in that low T6P does not put a brake on all metabolic pathways but may stimulate secondary metabolism, lipids in this case, and hence produce qualitative changes in metabolism. Despite similarities in gene expression between component parts of reproductive tissue, metabolite profiles showed contrasting patterns in pith and florets, with pith having reduced assimilate content and floret increased assimilate content (Fig. 5A, B, Supplementary Fig. 3A, B). This may be explained because pith is composed of soft spongy parenchyma cells that store and transport nutrients to the developing florets, which are terminal sinks for assimilate. Hence, whilst reproductive tissue is an overall sink for assimilate, within the developing cob, pith is a source of assimilate for florets. Effects of T6P are likely to be highly context specific. This is evident in the different response of Arabidopsis seedlings compared to rosettes, which are mainly sink tissue and source tissue, respectively (Zhang et al., 2009; Wingler et al., 2012). Previous studies have shown an association of trehalose pathway gene expression with vasculature (Ramon et al., 2009) and the expression of *Attps1* is high in phloem companion cells (Genevestigator, https://genevestigator.com/gv/). Vascular expression of *TPS1* resulted in early flowering, suggesting a biological significance of the pathway in vascular tissue (Ruiz-Salas et al., 2016). This, combined with the T6P-associated changes to assimilate distribution in phloem cells found in this study suggest that T6P may regulate the flow of assimilates between tissues such as the pith and floret of maize reproductive structures. Regulation of *SWEET* genes could be a key factor in the regulation of T6P-mediated assimilate transfer as it was the only major class of protein associated with assimilate transfer to be consistently affected in this study (Fig. 10). Five *SWEET* genes were upregulated in pith, potentially implicating them in the movement of sucrose from pith to floret (Fig. 10); *SWEET* genes are proposed to

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efflux sucrose to the phloem apoplasm (Braun et al., 2014). In particular, *SWEET13*, which was strongly affected in our study, has been previously singled out as a target for crop improvement in maize (Bezrutczyk et al., 2017). In explaining elevated amino acids in florets in the absence of consistent upregulation of amino acid synthesis or amino acid transport genes, it is possible that increased sucrose availability may have resulted in more carbon skeleton for the synthesis of amino acids. Allantoin was the only metabolite to be increased consistently in all tissues (Supplementary Fig. 3A, B) found to be associated with a decrease in transcript for allantoinase (Fig. 11). Allantoin is associated with nitrogen assimilation in legumes and is considered a transport form of nitrogen (Coneva et al., 2014; Collier and Tegeder, 2012). It is possible that this may be associated with changes in amino acids, although the mechanistic basis of this is not known. Allantoin accumulation is associated with a number of traits involved in the tolerance of stresses, such as cadmium and salt, through antioxidant mechanisms (Brychkova et al., 2008), and jasmonic acid and ABA signalling (Takagi et al., 2016).

Photosynthesis rate is maintained for longer in leaves of transgenics

The normal developmental decline of photosynthesis in leaves was slowed in the transgenics (Fig. 12). Since there was no evidence of expression of the transgene in leaves, explanations for elevated photosynthesis must come from elsewhere. It is possible that greater movement of sucrose from pith into florets increased demand for sucrose, which maintained a higher photosynthetic rate for longer. Upregulation of a number of *SWEET* genes in reproductive tissue, including node and shank, (Fig. 10) may have mediated the enhanced flow of sucrose from leaves. Sink regulation of photosynthesis has been a long-observed phenomenon (Paul and Foyer 2001). Recent work strongly implicates SWEET proteins in source–sink relationships in Arabidopsis (Durand et al., 2017). Sucrose levels in leaves of transgenics were also increased. This was consistent with increases in gene expression for sucrose biosynthesis and chloroplast processes in leaves (Fig. 13) and provides evidence of a wider role for T6P in source–sink relations regulating metabolism in sinks, which can provide feedforward regulation of leaf photosynthesis

T6P/ SnRK1-mediated regulation

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365   A second part of the current model of T6P's mode of action is that SnRK1 mediates

366   changes in gene expression associated with T6P abundance (Zhang et al., 2009;

367   Nunes et al., 2013). To better understand whether SnRK1 could mediate the effects

368   of lower T6P, expression of SnRK1 marker homologues was determined. Despite

369   the fact that over the long term, direct cause-and-effect correlations are likely to be

370   weakened by secondary and tertiary effects, there was evidence that changes in

371   T6P resulted in altered regulation of gene expression through SnRK1 as the SnRK1

372   marker genes normally induced by SnRK1 were induced (Fig. 7A) and those

373   repressed by SnRK1 were more repressed than wild type (Fig. 7B). SnRK1 was also

374   found to be inhibited by T6P and its activity, in the absence of T6P, was elevated in

375   transgenic pith and florets (Fig. 8). These data provide *in vivo* and *in vitro* evidence

376   of increased SnRK1 activity as a consequence of decreased T6P. This was

377   associated with altered expression of the gene for the SnRK1 beta subunit, which

378   may perform a regulatory rather than catalytic role within the SnRK1 complex

379   (Baena-Gonzalez and Hanson 2017). Changes in *AKIN11* expression, which

380   encodes the alpha subunit of the SnRK1 trimer were also found (Fig. 8). *AKIN11*

381   expression has previously been shown to be altered in Arabidopsis with altered T6P

382   (Schluepmann et al., 2004). Coincident with changes in SnRK1 was significantly less

383   expression of endogenous trehalose pathway genes (Fig. 9). It was found previously

384   that elevated T6P in Arabidopsis caused changes in expression of trehalose

385   pathway genes, particularly class II TPSs (Zhang et al., 2009) in accordance with

386   them being starvation inducible and sucrose repressible (Nunes et al., 2013a; Yadav

387   et al., 2014). Here, these genes were induced in pith and induced to a lesser extent

388   in florets. Our data confirm strong overlap and/or strong direct convergence between

389   the SnRK1 and T6P signalling pathways, with the T6P inhibition of SnRK1 activity as

390   a putative mechanism of metabolic reprogramming.

391   An association between T6P and sucrose is well established (Lunn et al.,

392   2006; Nunes et al., 2013a). In this study, decreased T6P in transgenic pith correlated

393   with less sucrose in pith, but less T6P in transgenic florets correlated with more

394   sucrose. This confirms that the sucrose: T6P nexus (Yadav et al., 2014) is tissue

395   dependent. The effect of T6P on the altered distribution and expression of *SWEET*

396   genes implies a broader role of T6P in movement of sucrose between tissues.

397

398   **Summary**

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399 The data in this study support a model wherein decreased T6P downregulates
400 primary metabolism but upregulates secondary metabolism in the form of lipid
401 synthesis. Low T6P resulted in different metabolite profiles in pith and floret with
402 evidence that T6P regulates the movement of assimilate from pith to florets. This
403 movement of sucrose may create extra demand for sucrose from leaves, giving rise
404 to maintenance of high photosynthetic rates for longer. This indicates that T6P can
405 regulate the balance between primary and secondary metabolism, the balance of
406 assimilate accumulation within component parts of reproductive tissues and the
407 activity of photosynthesis. Hence, changes in T6P in sinks can act as a major
408 regulator of whole plant source/sink balance. Overall the changes in metabolite
409 profiles and gene expression patterns in transgenics were similar under well-watered
410 and drought conditions. This similarity indicates that these transgenics are
411 predisposed to cope with drought and yield better under well-watered conditions by
412 maintaining a source–sink balance that favours sucrose allocation to florets. Further,
413 the data indicate that low T6P can upregulate secondary metabolism in the form of
414 lipid synthesis. A strategy to target T6P in this way would improve both crop yields in
415 different environments and produce qualitative changes in secondary metabolic
416 pathways such as lipid biosynthesis.

417

418 **METHODS**

419 **Plant Material, Growth Conditions and Sampling**

420 Two independent transgenic lines (5217 and 5224) of maize (*Zea mays*) expressing
421 the *OSMADS6: TPP1* construct, wild-type line A188 and *OSMADS6: GUS* were
422 grown in a controlled environment (600 µmol m^{-2} s^{-1}, 16 h day, 80/70% relative
423 humidity day/night, 27°C/21°C day/night in Rothamsted compost supplemented with
424 full nutrition as in Nuccio et al., 2015). Five different tissue types from female
425 reproductive tissue (florets, shank, pith, node and fully expanded leaf next to the
426 developing cob) were sampled at 5-day intervals at four time points, 5 days before
427 pollination to 10 days after pollination (labelled as 1, 2, 3, 4; corresponding to -5, 0,
428 5, 10 days relative to pollination). Five biological replicates from individual plants
429 were taken for each line, time and tissue. Tissue was sampled during the middle of
430 the photoperiod, snap frozen in liquid N$_2$ and stored at -80°C until analysis. Plants
431 were grown under full irrigation, or with drought imposed 5 days before the first
432 sampling point by withholding water until pots reached 65% of the weight before

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433 withholding water, as in Nuccio et al. (2015). This level of drought was maintained
434 throughout the sampling period by weighing plants daily. *OSMADS6: GUS*
435 expression cassette activity was assessed by histochemical localisation of β-
436 glucuronidase (GUS) protein (described in Nuccio et al., 2015).

437

438 **RNA seq**
439 Total RNA was extracted from 100 mg ground maize tissue using the Ribopure™ Kit
440 (Ambion®) according to the manufacturer's instructions. RNA was quantified using a
441 Nanodrop and RNA integrity quality was checked by Agilent RNA 6000 Nano Kit®
442 (Agilent Technology™) according to the manufacturer's instructions.

443 Sequence libraries were prepared at Syngenta and samples were sequenced
444 on a HiSeq2000 by GeneWiz. FastQ sequence files were examined by FastQC
445 software (v0.11.2). Raw reads were used for mapping to the maize reference
446 genome 5a57 (AGP_v2) with gene models 5b.60 (www.maizegdb.org). Tophat
447 (v2.0.12) with bowtie2 (version 2.2.3) was used for read alignment (Trapnell, 2009;
448 Langmead, 2009). The maximum read mismatches allowed were six. Reads which
449 mapped to more than six places were randomly reported to six mapped locations.
450 Counts were generated from alignment files in BAM format using a custom Python
451 script. Briefly, the method produces counts for gene regions that are defined by a
452 gene feature file (gff3 format) for reads aligning to that region. If a given read aligned
453 to multiple genomic regions defined as genes, up to six regions received one count
454 each. If the read aligned to more than six genomic loci defined as genes, then the
455 read was discarded. This method avoided loss of useful information in cases where
456 reads aligned equally well to different genes. The annotation file used for gene count
457 generation was customized to include sequences for the trait gene *Tpp1* and the
458 phosphomannose isomerase (PMI) selectable marker gene used in transformation,
459 as well as maize mitochondria and chloroplast sequences. A further step included
460 filtering by raw counts before any normalization. Genes were only kept if they had a
461 'counts per million' value of 10 or more in at least three different samples which
462 resulted in a total of 22,714 and 22,748 features (genes) reported for the unstressed
463 and drought studies, respectively.

464 Read quality was analysed using FastQC
465 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Most of the samples
466 had fairly similar GC content, consistent with the GC content of the Maize genome.

14

467 To correct for possible GC or length bias, data were normalized using the EDASeq R

468 package (Risso, 2011). Differential expression analysis and gene ontology (GO)

469 term and pathway enrichment analyses were conducted using the EdgeR package

470 (Robinson et al., 2010).

471 **GO term and Pathway Enrichment Analysis**

472 Gene expression analysis from RNAseq was summarised into genes significantly

473 increased or decreased in both transgenic lines compared to wild type in all tissues

474 under well-watered and drought conditions (Fig. 4, Fig. 6, Supplementary Tables S1-

475 4). Genes were considered to be differentially expressed if results of statistical tests

476 had a false discovery rate of less than 0.05 and the effect size, measured as

477 absolute value of log2 fold change, was greater than one. The experimental design

478 resulted in a total of 80 contrasts of transgenic lines compared to wild type controls

479 across the five tissue types, four time points and different watering regimes. Initial

480 contrasts compared individual transgenic events to wild type controls. Roast

481 implemented in the EdgeR package was used for enrichment of GO terms and sets

482 of genes predicted to code for enzymes in biochemical pathways (Wu et al., 2010).

483 Roast uses a gene set test that assigns a P-value to a set of genes as a unit, which

484 increases statistical power for interpreting results compared with other available

485 permutation tests or more traditional methods such as Fisher's Exact tests for gene

486 set enrichment. Gene sets of biochemical pathways and GO terms were assembled

487 from data obtained from the Maize Genetics and Genomics Database

488 (www.maizeGDB.org). For pathway data, BioPax formatted files were downloaded

489 from MaizeCyc2.0.1 and parsed to get all gene models likely encoding enzymes in

490 all maize biochemical pathways and reactions in the data set. These data and GO

491 annotations were formatted into files suitable for use with Roast using ad hoc scripts.

492 Data from mRoast, a version of Roast for multiple gene sets, included multiple test

493 corrected P-values (False Discovery Rate (FDR)) as well as directionality of change

494 of the majority of genes within a defined gene set. For Figure 6, data were

495 condensed by first only reporting pathways significantly enriched (false discovery

496 rate of < 0.05) in the same direction by both transgenic events in a particular time

497 point and tissue. This was done for both unstressed and drought stressed plants.

498 Secondly, pathways were only reported if they were significantly perturbed in the

499 same direction in both transgenic events in at least two of the four time points tested.

15

These pathway results were further condensed using the pathway ontology in MaizeCyc2.0.1. Individual pathways were grouped together into higher order biochemical pathways using these ontologies. Only pathways included in this ontology were included, all individual reactions enriched in the data set were eliminated. In cases where multiple pathways were present under a given pathway ontology, a single pathway was chosen as most representative of the pathway category.

Metabolite Analysis

All extraction and analysis was conducted at Metabolon. Samples were extracted and split into equal parts for analysis on GC/MS and LC/MS/MS platforms. Proprietary software was used to match ions to an in-house library of standards for metabolite identification and quantitation by peak area integration. LC/MS was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer consisting of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion. Samples for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp 40°C to 300°C over 16 min. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy daily. The information output from the raw data files was automatically extracted as discussed below. Accurate mass determination (LC/MS) and MS/MS fragmentation (LC/MS/MS) for structural elucidation was based on a Waters ACQUITY UPLC and a Thermo-Finnigan OrbiElite mass spectrometer, which had an LIT front end and an

16

534 orbitrap mass spectrometer back end. Accurate mass measurements could be made
535 on the parent ion as well as fragments. The typical mass error was less than 5 ppm.

536   The informatics system consisted of four major components, the Laboratory
537 Information Management System (LIMS), the data extraction and peak-identification
538 software, data processing tools for QC and compound identification, and a collection
539 of information interpretation and visualization tools for use by data analysts. The
540 hardware and software foundations for these informatics components were the LAN
541 backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition. Peaks
542 were identified using Metabolon's proprietary peak integration software, and
543 component parts were stored in a separate and specifically designed complex data
544 structure. Compounds were identified by comparison to library entries of purified
545 standards or recurrent unknown entities.

546   For statistical analysis pair-wise comparisons using Welch's T-tests and/or
547 Wilcoxon's rank sum tests were performed. ANOVA was also conducted.
548 Metabosync (Metabolon Inc, Durham, NC, USA) was used to show changes in
549 metabolite abundance in representative contrasts of transgenic event 5224
550 compared to wild type grown in unstressed conditions to show the full extent of
551 metabolite changes using Welch's T-tests to calculate the size of the circle and
552 statistical significance of difference of mean values (Fig. 5A, B).

553

554 **SnRK1 Activity**
555 Total soluble protein was extracted from 200 mg of tissue ground under liquid
556 nitrogen in a pestle and mortar in 600 µL of ice-cold homogenization buffer of 100
557 mM tricine-NaOH, pH 8, 25 mM NaF, 5 mM dithiothreitol, 2 mM tetrasodium
558 pyrophosphate, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM benzamidine, 1 mM
559 phenylmethylsulfonyl fluoride, 1 mM protease inhibitor cocktail (Sigma P9599),
560 phosphatase inhibitors (PhosStop; Roche) and insoluble polyvinylpyrrolidone to 2%
561 (w/v). Homogenate was centrifuged at 13,000g at 4ºC. Supernatant (250 µL) was
562 desalted in illustra NAP-5 columns (GE Healthcare) pre-equilibrated with
563 homogenization buffer. Eluent was supplemented with protease inhibitor cocktail and
564 okadaic acid to 2.5 mM before freezing in liquid nitrogen. SnRK1 activity of three
565 replicates for each time point was determined as described by Zhang et al. (2009) in
566 a final volume of 25 µl in microtitre plate wells at 30ºC. Assay medium was 40 mM

17

567 HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 200 mM ATP containing 12.5 kBq [γ-^{33}P] ATP

568 (PerkinElmer), 200 µM AMARA peptide (Enzo Life Sciences, UK, Ltd), 5 mM

569 dithiothreitol, 1 µM okadaic acid and 1 mM protease inhibitor cocktail (Sigma P9599).

570 Assays were started with 5 µL extract and stopped after 6 min by transferring 15 µL

571 to 4 cm^2 squares of Whatman P81 phosphocellulose paper immersed immediately in

572 1% phosphoric acid. These were then washed with four 800 ml volumes of 1%

573 phosphoric acid, immersed in acetone for 15 min, air dried and transferred to vials

574 with 3.5 ml of scintillation cocktail (Ultima Gold).

575

576 **Photosynthesis**

577 Leaf gas exchange measurements of well-watered and water-stressed maize plants

578 were carried out using a portable infra-red open gas exchange system (LI-6400XT;

579 LI-COR, Lincoln, USA) under the growing conditions (ambient CO_2 (400 µl l^{-1}), leaf

580 temperature 27°C, photosynthetic photon flux density 600 µmol m^{-2} s^{-1}, relative air

581 humidity 65 ± 5%. Each leaf reached a steady state of CO_2 uptake in the leaf

582 chamber before measurements were taken.

583 **ACCESSION NUMBERS**

584 Maize SnRK1 gene accession numbers: SnRK1beta, GRMZM2G025459; AKINB,

585 GRMZM2G138814; AKIN10B, GRMZM2G077278; AKIN10, GRMZM2G077278;

586 AKIN11, GRMZM2G107867.

587 Maize trehalose pathway gene accession numbers: TPPA1, GRMZM2G178546;

588 TPPB1.3, GRMZM2G174396; TPPA.3, GRMZM2G112830; TPPB.1.2,

589 GRMZM2G140078; TPPB.1.4, GRMZM2G055150; TPSII.3.2, GRMZM2G123277;

590 TPSII.4.2, GRMZM2G008226; TPSII.4.1GRMZM2G527891; TPSII.2.1,

591 GRMZM2G019183; TPSII.5.4, GRMZM2G122231; TPSII.4.3, GRMZM2G366659;

592 TPSII.5.3, GRMZM2G312521; TPPB.2.1, GRMZM2G014729.

593 Maize SWEET gene accession numbers: SWEET1b, GRMZM2G153358; SWEET2,

594 GRMZM2G324903; SWEET3a, GRMZM2G179679; SWEET11, GRMZM2G368827;

595 SWEET13a, GRMZM2G173669; SWEET13c, GRMZM2G179349; SWEET14b,

596 GRMZM2G015976; SWEET15a, GRMZM2G168365; SWEET15b,

597 GRMZM5G972392; SWEET16, GRMZM2G107597.

598 Maize allantoinase gene accession number: GRMZM2G173413.

599 RNAseq Accession: PRJNA421180 ID: 421180; Unstressed samples: SUB3315967;

600 Drought Stressed Samples: SUB3357197

601 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA421180

602

603 **SUPPLEMENTAL DATA**

604 **Supplementary Figure S1. Histochemical analysis of *OSMADS6: GUS*.**

605 **Supplementary Figure S2. Histochemical analysis of *OSMADS6: GUS* activity**

606 **in node.**

607 **Supplementary Figure S3. Effect of *OSMADS6: TPP1* on selected metabolites**

608 **in reproductive tissues and leaves during early reproductive development.**

609 **Supplementary Table S1. Changes in gene expression.**

610 **Supplementary Table S2. Changes in gene expression.**

611 **Supplementary Table S3. Changes in gene expression.**

612 **Supplementary Table S4. Changes in gene expression.**

613 **Supplementary Table S5. SnRK1 marker gene expression previously identified**

614 **in Baena-Gonzalez et al. (2007); Zhang et al. (2009); Martinez-Barajas et al.**

615 **(2011) in transgenics compared to wild type.**

616 **Supplementary Table S6. SnRK1 gene expression in transgenics compared to**

617 **wild type.**

618 **Supplementary Table S7. Trehalose pathway gene expression in transgenics**

619 **compared to wild type.**

620 **Supplementary Table S8. SWEET gene expression in transgenics compared to**

621 **wild type.**

622

627

FIGURE LEGENDS

Figure 1. *OSMADS6: TPP1* **expression in reproductive tissues and leaves during early reproductive development.**

Data are from (**A**) unstressed and (**B**) drought-stressed plants. Normalized gene count data are plotted for each transgenic event (5217 or 5224) and the wild-type (WT) A188 line. The horizontal line in each box indicates the mean. Vertical lines indicate the range for each sample. The time points are 5 days before pollination (-5), day of pollination (0), 5 days after pollination (5) and 10 days after pollination (10). Data are the mean +/- SD of 5 biological replicates.

Figure 2. Histochemical localization of β-glucuronidase activity produced by the *OSMADS6: GUS* **reporter gene in unstressed maize during early reproductive development.**

Samples were collected from **(A-D)** ear florets, **(E-H)** shank, **(I-L)** node, **(M-N)** shank at high magnification, **(O)** node at high magnification, **(P)** pith at high magnification. Samples were collected at 5 days before pollination **(A, E, I, M)**, day of pollination **(B, F, J, N)**, 5 days after pollination **(C, G, K, O)** and 10 days after pollination **(D, H, L, P)**. Samples were incubated in the histochemical reagent and cleared as described in the Methods. Xylem and phloem cells are indicated by the red arrows.

Figure 3. Effect of the *OSMADS6: TPP1* **on T6P and trehalose in reproductive tissues and leaves during early reproductive development.**

Samples were collected from **(A, C)** unstressed and **(B, D)** drought-stressed plants at 5 days before pollination (-5), day of pollination (0), 5 days after pollination (5) and 10 days after pollination (10). Vertical lines indicate the range for each sample. Data are the mean ± SD (n=5).

Figure 4. Screen shot from Metabosync showing change in metabolite abundance in response to *OSMADS6: TPP1*. Representative contrasts of transgenic event 5224 compared to wild type grown in unstressed conditions to show full extent of metabolite changes. **A.** Floret tissue at day of pollination. **B.** Pith tissue 5 days after pollination. Biochemicals shown are key metabolites in aromatic amino acid metabolism, glutamate family amino acid synthesis, phospholipid metabolism,

20

661 sucrose metabolism, aspartate family amino acid synthesis, arginine biosynthesis,

662 BCAA metabolism, trehalose biosynthesis and nitrogen metabolism. Circles

663 represent metabolites as labeled. The size of the circle indicates statistical

664 significance of difference of mean values from Welch's T-tests. Blue indicates fold

665 change (ratio of mean value from all replicates in samples of transgenic event/ wild

666 type) of less than one and red indicates fold change of greater than one.

667

668 **Figure 5.** Genes significantly upregulated or downregulated in different tissues in

669 both *OSMADS6: TPP1* transgenic lines compared to wild type in well-watered

670 conditions and under drought. The time points are 5 days before pollination (1), day

671 of pollination (2), 5 days after pollination (3), and 10 days after pollination (4)

672

673 **Figure 6. *OSMADS6: TPP1* effect on biochemical pathways.** Transcript profiling

674 data from both the 5217 and 5224 events were compared to wild type (A188) to

675 identify significantly perturbed biochemical pathways. A white cell indicates pathways

676 that were not affected and a dark cell (red, up; blue, down) indicates pathways that

677 were significantly affected by the transgene. The data are from unstressed (US) and

678 drought stressed (DS) plants. Results from the four time points were condensed by

679 only reporting pathways significantly affected in at least two time points.

680

681 **Figure 7. Heat map showing the effect of *OSMADS6: TPP1* on SnRK1-regulated**

682 **genes.** Maize orthologues of Arabidopsis/ wheat genes shown to be **(A)** induced or

683 **(B)** suppressed by SnRK1 in Arabidopsis/ wheat (Zhang et al., 2009; Martinez-

684 Barajas et al., 2011) were examined using differential expression analysis from

685 unstressed (US) or drought-stressed (DS) plants between wild type and transgenic

686 lines 5217 and 5224. Red indicates upregulation and blue indicates downregulation

687 relative to wild type (A188). White indicates no significant (NS) difference and grey

688 indicates no data. The time points are 5 days before pollination (1), day of pollination

689 (2), 5 days after pollination (3) and 10 days after pollination (4).

690

691 **Figure 8. Effect of *OSMADS6: TPP1* on SnRK1 activity and gene expression.**

692 Pith and floret tissues were examined for **(A)** extractable SnRK1 activity (line 5127)

693 and **(B)** differential expression of maize orthologues of Arabidopsis genes that

694 encode SnRK1 subunits between wild type and transgenic line 5217 and 5224.

21

SnRK1 activity was assayed in the presence and absence of 1 mM T6P. Data are the mean ± SD (n = 3). The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

Figure 9. Effect of *OSMADS6: TPP1* on trehalose metabolism gene expression. Differential expression analysis of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) gene family members between wild type and transgenic lines 5217 and 5224. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

Figure 10. Heat map showing the effect of *OSMADS6: TPP1* on SWEET genes. Heat maps represent differential expression analysis between wild type and transgenic lines 5217 and 5224. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

Figure 11. Heat map showing allantoinase and nitrogen transport and metabolic processes. Heat maps represent differential expression analysis between wild type and transgenic lines 5217 and 5224. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

Figure 12. Rates of photosynthesis during 2-week flowering period. Wild type (open symbols) and *OSMADS6: TPP1* transgenic line 5224 (closed symbols). **(A, B)** the leaf next to the ear and **(C, D)** the leaf above the ear. Unstressed **(A, C)** and drought stressed **(B, D)**. Time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3), 10 days after pollination (4). Rates of CO_2 uptake were measured under the growing conditions (ambient CO_2 (400 µl l^{-1}), leaf temperature 27°C, photosynthetic photon flux density 600 µmol m^{-2} s^{-1}, relative air humidity 60 ± 5%. Data are the mean ± SD (n = 4). * indicates statistical significance between transgenic and wild type at $p < 0.05$.

Figure 13. Heat map showing photosynthesis genes in mature leaves.

22

727 Heat maps represent differential expression analysis between wild type and

728 transgenic lines 5217 and 5224. The time points are 5 days before pollination (1),

729 day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

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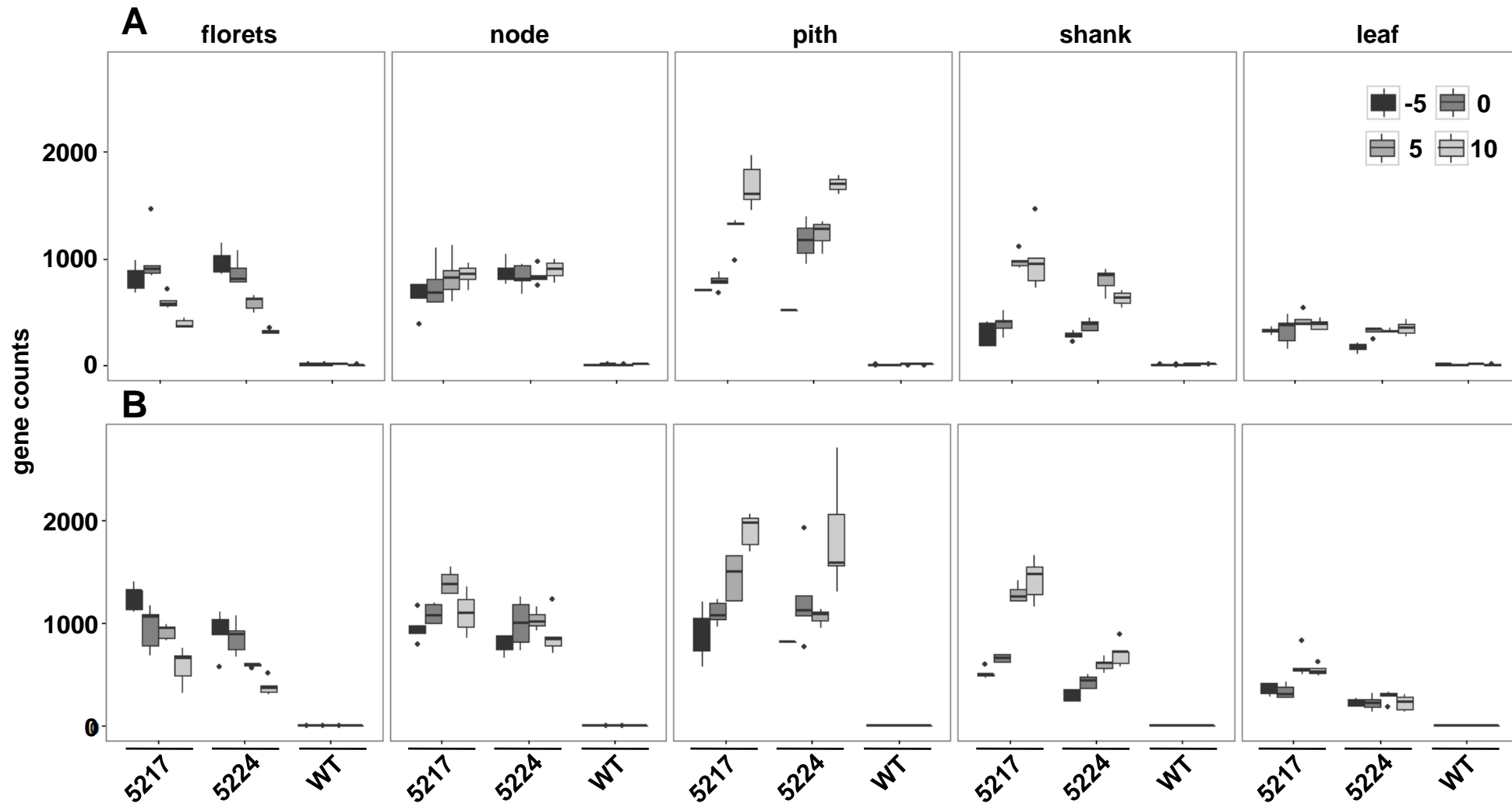
Figure 1. *OSMADS6: TPP1* expression in various maize tissues during early reproductive development. Data are from **(A)** unstressed and **(B)** drought stressed plants. Normalized gene count data are plotted for each transgenic event (5217 or 5224) and the wild-type (WT. The horizontal line in the each box indicates the mean. Vertical lines indicate the range for each sample. The time points are 5 days before pollination (-5), day of pollination (0), 5 days after pollination (5), and 10 days after pollination (10). Data are the mean +/- SD of 5 biological replicates
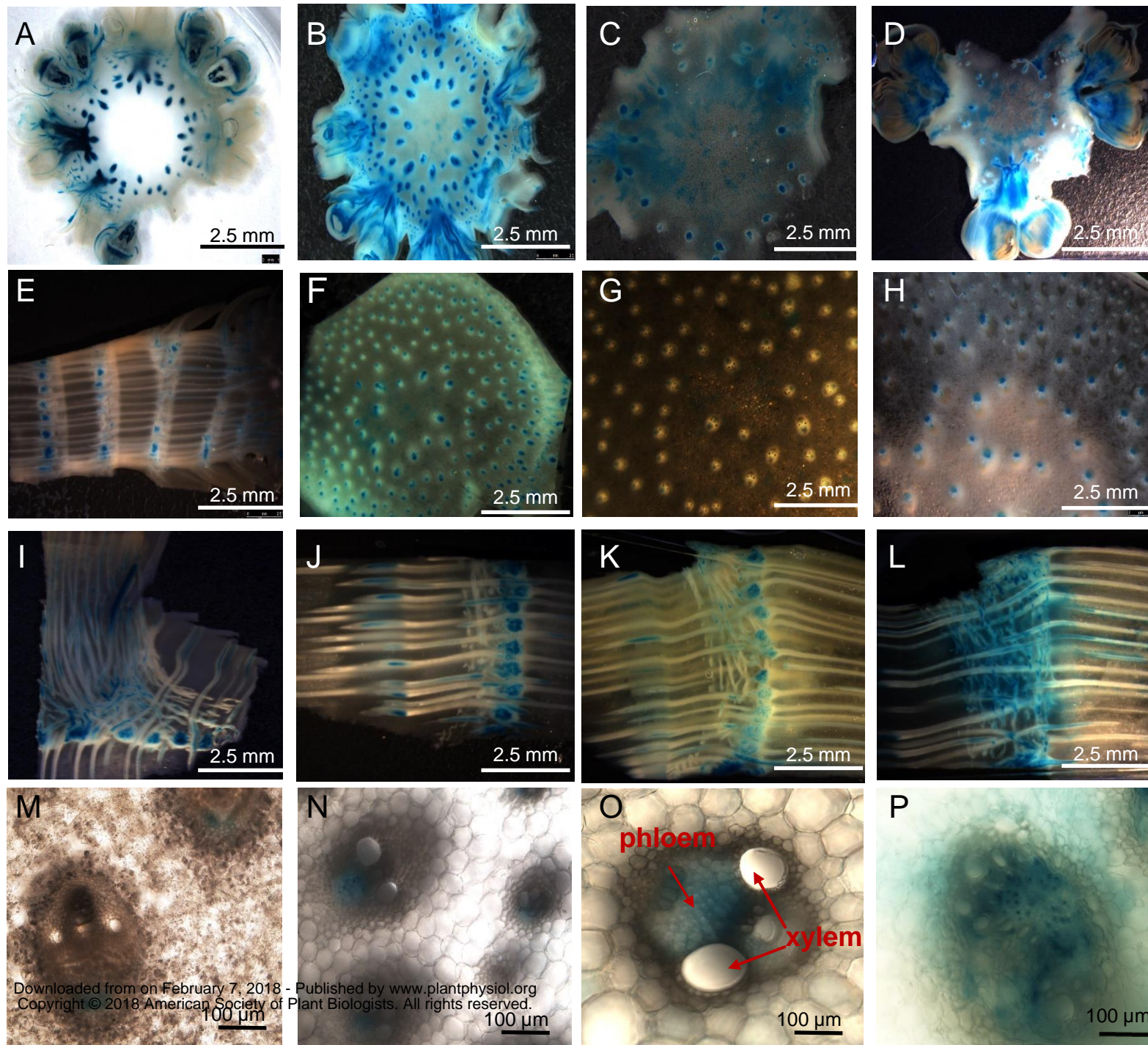
Figure 2. Histochemical localization of β-glucuronidase activity produced by the OsMads6-GUS reporter gene in unstressed maize during early reproductive development. Samples were collected from (**A**-**D**) ear spikelets, (**E**-**H**) shank, (**I**-**L**) node, (**M**-**N**) shank at high magnification, (**O**) node at high magnification, (**P**) pith at high magnification. Samples were collected at 5 days before pollination (**A**, **E**, **I**, **M**), day of pollination (**B**, **F**, **J**, **N**), 5 days after pollination (**C**, **G**, **K**, **O**) and 10 days after pollination (**D**, **H**, **L**, **P**). Samples were incubated in the histochemical reagent and cleared as described in the Methods. Xylem and phloem cells are indicated by the red arrows.
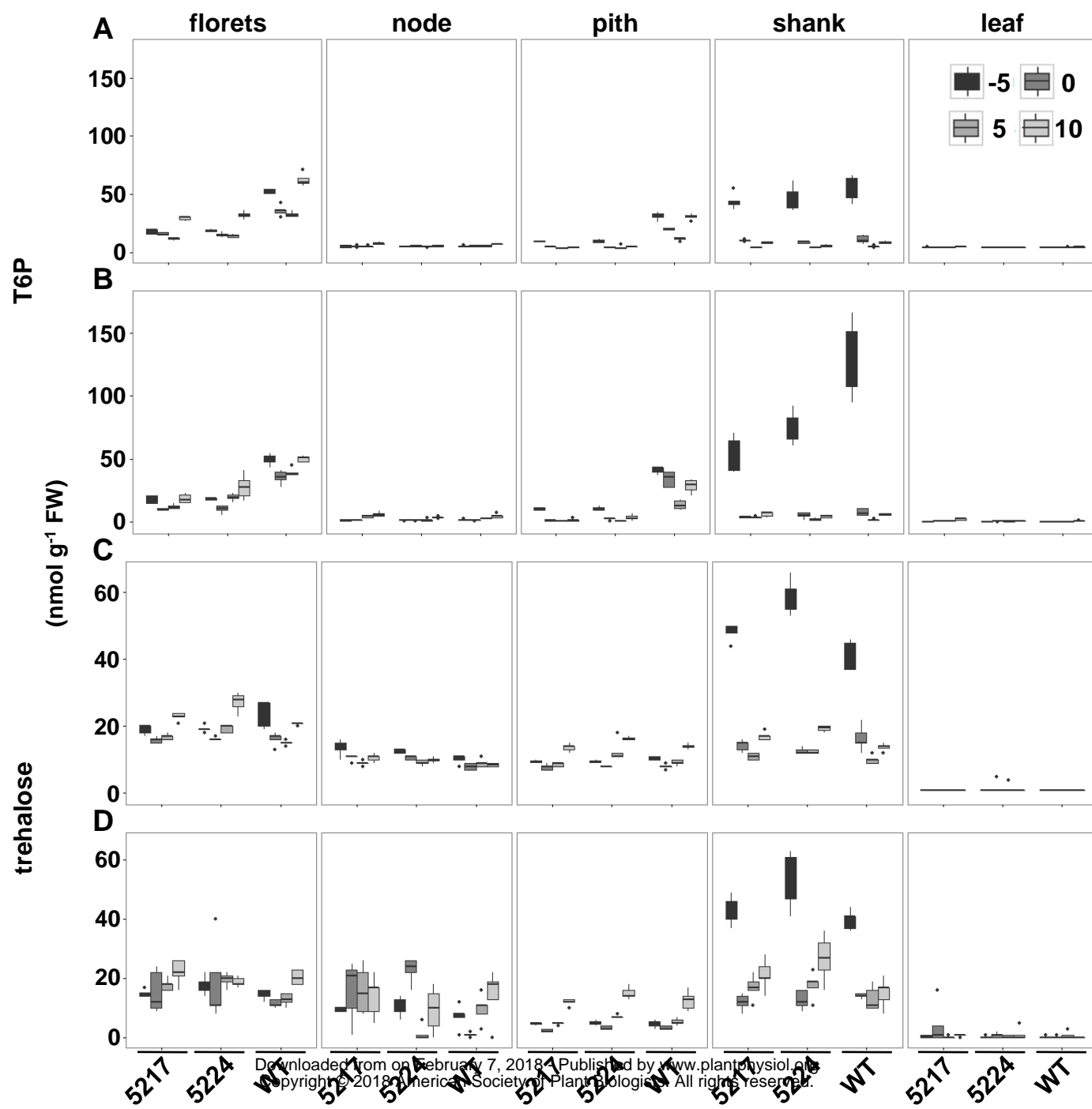
Figure 3. Effect of the *OSMADS6: TPP1* on T6P and trehalose in various tissues during early reproductive development comparing lines 5217, 5224 with wild type (WT). Samples were collected from **(A, C)** unstressed and **(B, D)** drought stressed plants. The time points are as in Figure 1. Data are the mean ± SD (n=4).
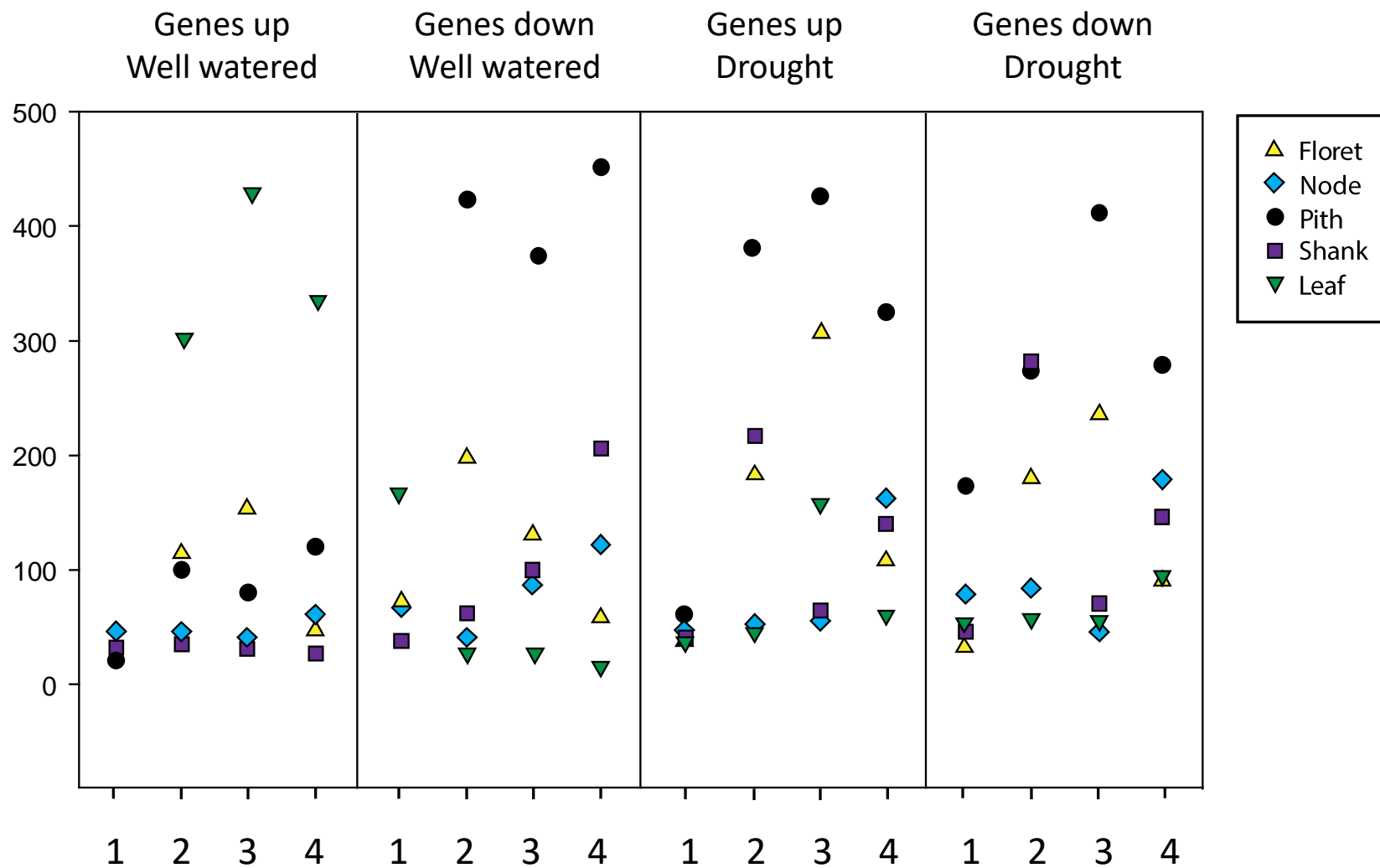
Figure 4. Screen shot from Metabosync showing change in metabolite abundance in response to *OSMADS6: TPP1*. Representative contrasts of transgenic event 5224 compared to wild type grown in unstressed conditions to show full extent of metabolite changes. **A.** Floret tissue at day of pollination. **B.** Pith tissue 5 days after pollination. Biochemicals shown are key metabolites in aromatic amino acid metabolism, glutamate family amino acid synthesis, phospholipid metabolism, sucrose metabolism, aspartate family amino acid synthesis, arginine biosynthesis, BCAA metabolism, trehalose biosynthesis and nitrogen metabolism. Circles represent metabolites as labeled. The size of the circle indicates statistical significance of difference of mean values from Welch's T-tests. Blue indicates fold change (ratio of mean value from all replicates in samples of transgenic event/ wild type) of less than one and red indicates fold change of greater than one.

Figure 4. Screen shot from Metabosync showing change in metabolite abundance in response to *OSMADS6: TPP1*. Representative contrasts of transgenic event 5224 compared to wild type grown in unstressed conditions to show full extent of metabolite changes. **A.** Floret tissue at day of pollination. **B.** Pith tissue 5 days after pollination. Biochemicals shown are key metabolites in aromatic amino acid metabolism, glutamate family amino acid synthesis, phospholipid metabolism, sucrose metabolism, aspartate family amino acid synthesis, arginine biosynthesis, BCAA metabolism, trehalose biosynthesis and nitrogen metabolism. Circles represent metabolites as labeled. The size of the circle indicates statistical significance of difference of mean values from Welch's T-tests. Blue indicates fold change (ratio of mean value from all replicates in samples of transgenic event/ wild type) of less than one and red indicates fold change of greater than one.
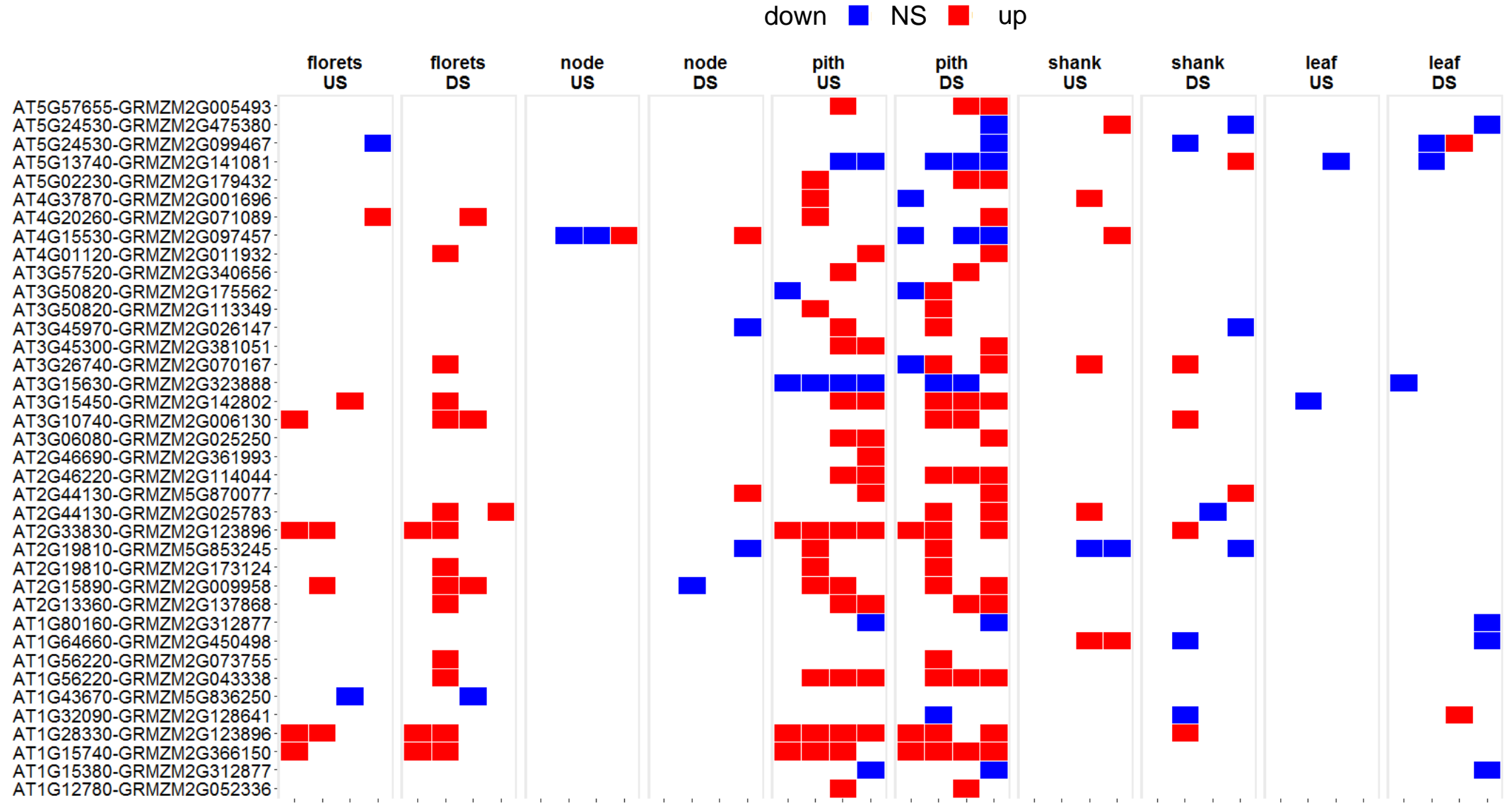
Figure 5. Genes significantly upregulated or down regulated in different tissues in both *OSMADS6: TPP1* transgenic lines compared to wild type in well-watered conditions and under drought. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3), and 10 days after pollination (4).
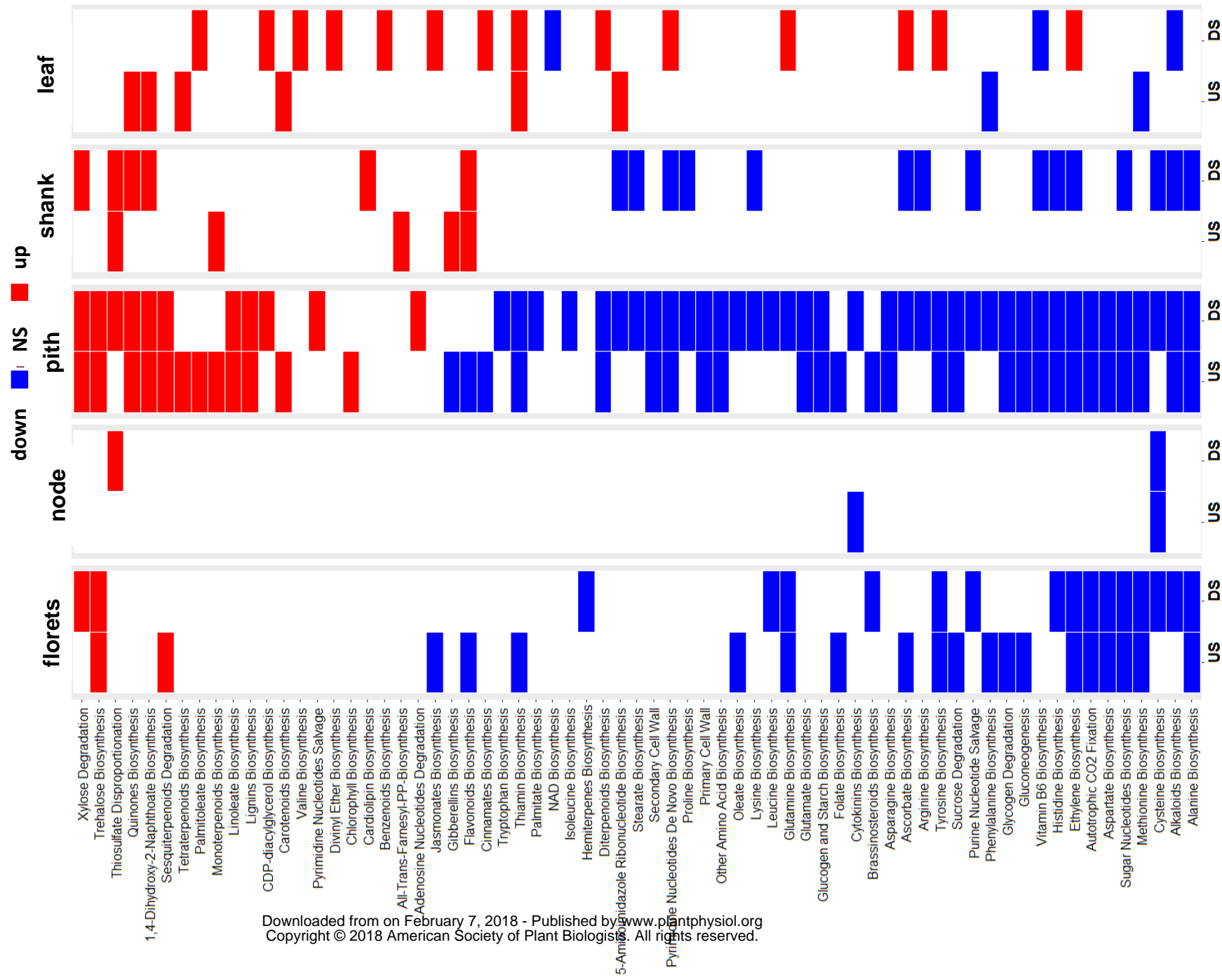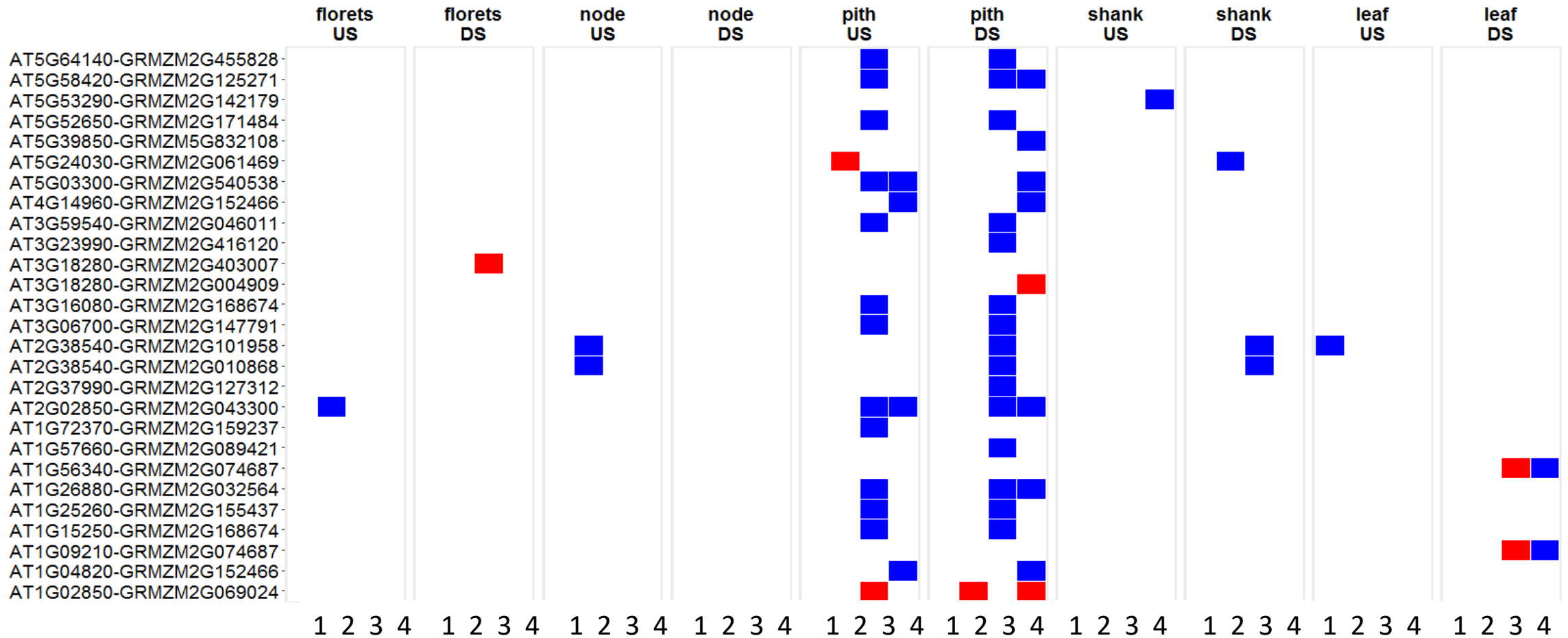
Figure 6. *OSMADS6: TPP1* effect on biochemical pathways in maize. Transcript profiling data from both the 5217 and 5224 events were compared to wildtype to identify significantly perturbed biochemical pathways. A white cell indicates pathways that were not affected and a dark cell indicates pathways that were significantly affected by the transgene. The data are from unstressed and drought stressed plants. Results from the four time points were condensed by only reporting pathways significantly affected in at least two time points.

**Figure 7.** Heat map showing the *OSMADS6: TPP1* effect on SnRK1 regulated genes. Transcript profiling data from both the 5217 and 5224 events compared to wildtype. The *OsMads6-Tpp1* effect on maize orthologs of Arabidopsis/wheat genes shown to be **(A)** induced by SnRK1 using differential expression analysis. The data represent the indicated tissues harvested from unstressed (US) or drought stressed (DS) plants. Red indicates indicates up-regulation and blue indicates down-regulation relative to wildtype (A188). White indicates no significant (NS). Time points shown on the x-axis are 1, 5 days before pollination; 2, Day of pollination; 3, 5 days after pollination; 4, 10 days after pollination.
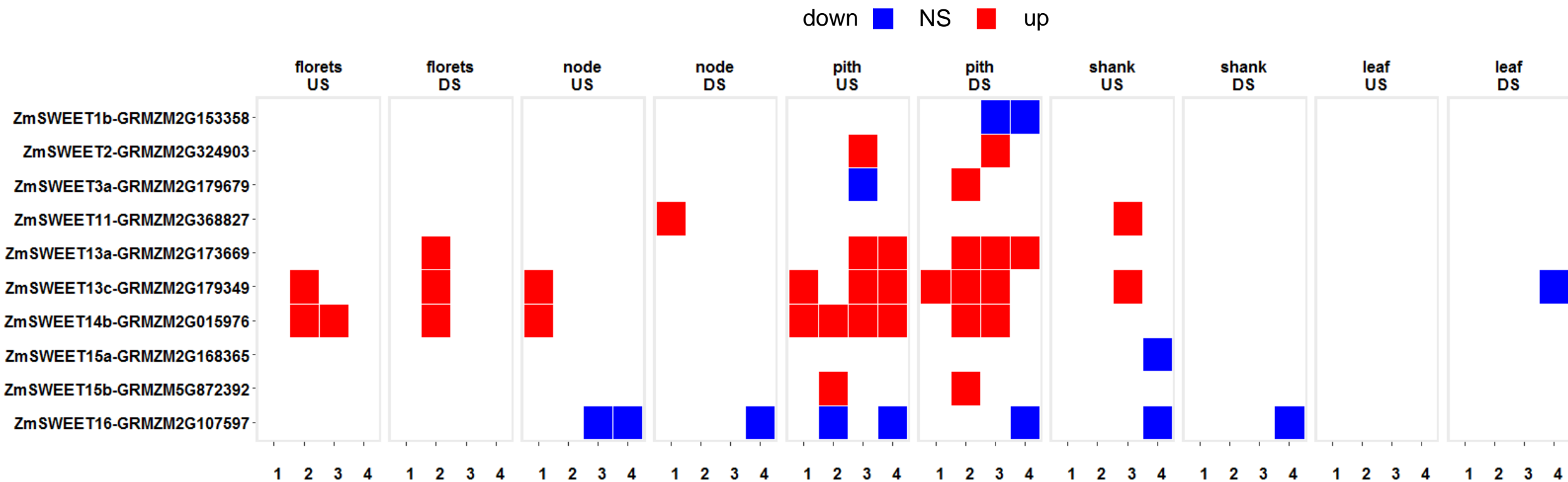
Figure 7. Heat map showing the *OSMADS6: TPP1* effect on SnRK1 regulated genes. Transcript profiling data from both the 5217 and 5224 events compared to wildtype. The *OsMads6-Tpp1* effect on maize orthologs of Arabidopsis/wheat genes shown to be **(B)** induced by SnRK1 using differential expression analysis. The data represent the indicated tissues harvested from unstressed (US) or drought stressed (DS) plants. Red indicates indicates up-regulation and blue indicates down-regulation relative to wildtype (A188). White indicates no significant (NS). Time points shown on the x-axis are 1, 5 days before pollination; 2, Day of pollination; 3, 5 days after pollination; 4, 10 days after pollination.
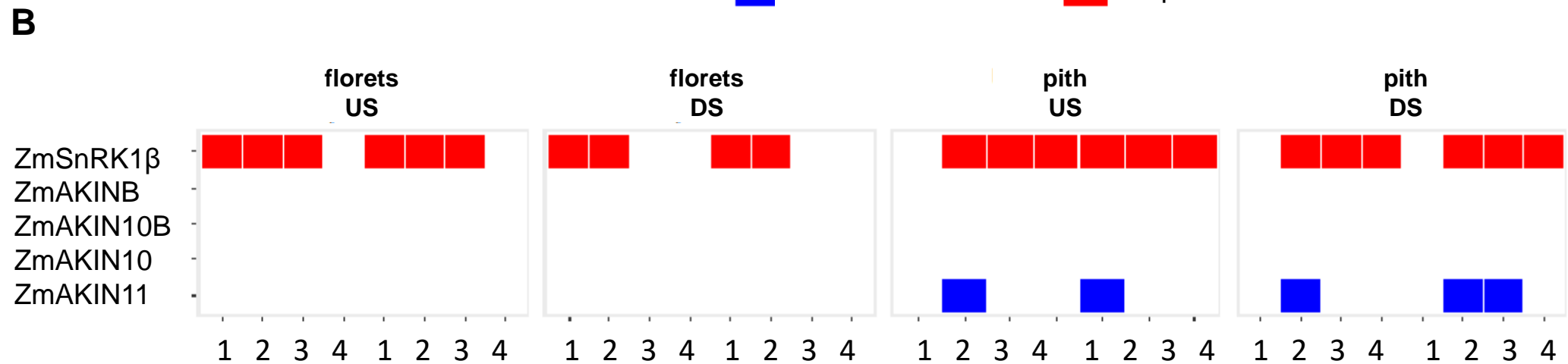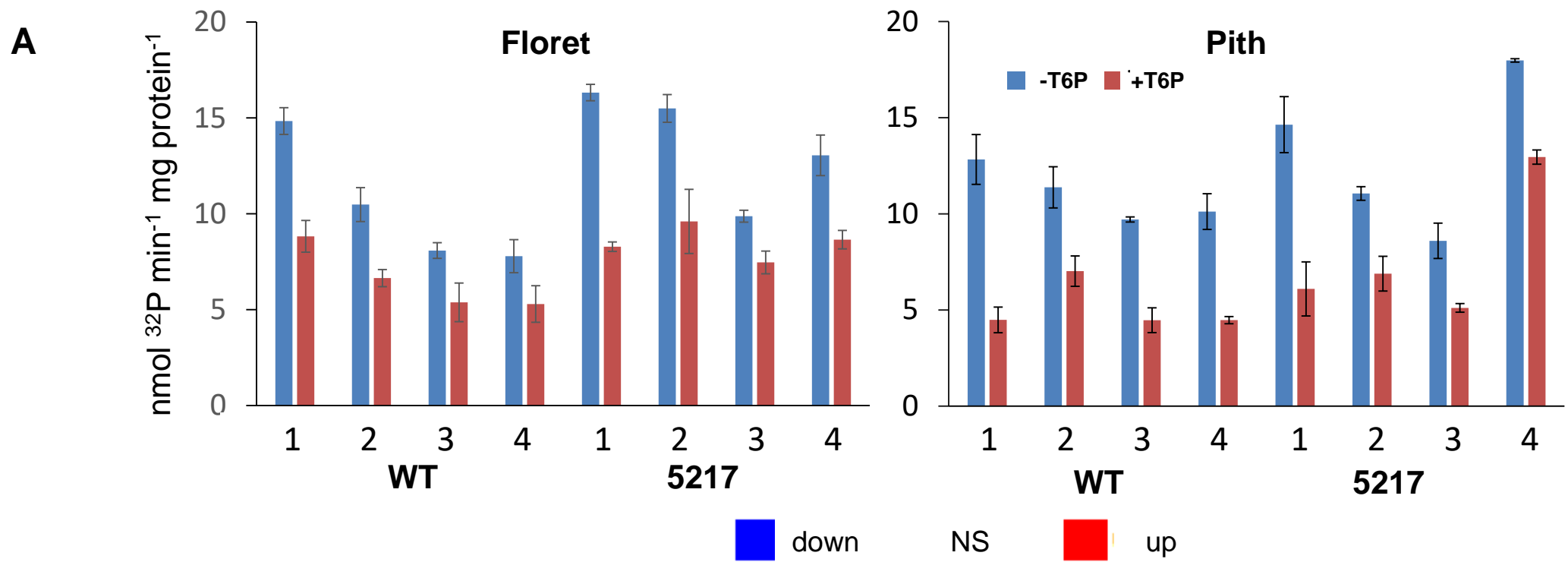
Figure 8. *OSMADS6: TPP1* effect on SnRK1 activity. Pith and floret tissues were examined for (A) Extractable SnRK1 activity (line 5217) and (B) effect on maize orthologs of Arabidopsis genes that encode SnRK1 subunits (lines 5217, 5224). SnRK1 activity was assayed in the presence and absence of 1 mM T6P. Data are the mean ± SD (n = 3). The maize genes are ZmSNRK1β, GRMZM2G025459; ZmAKINB, GRMZM2G138814; ZmAKIN10B, GRMZM2G077278; ZmAKIN10, GRMZM2G077278; ZmAKIN11, GRMZM2G107867. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3), and 10 days after pollination (4).
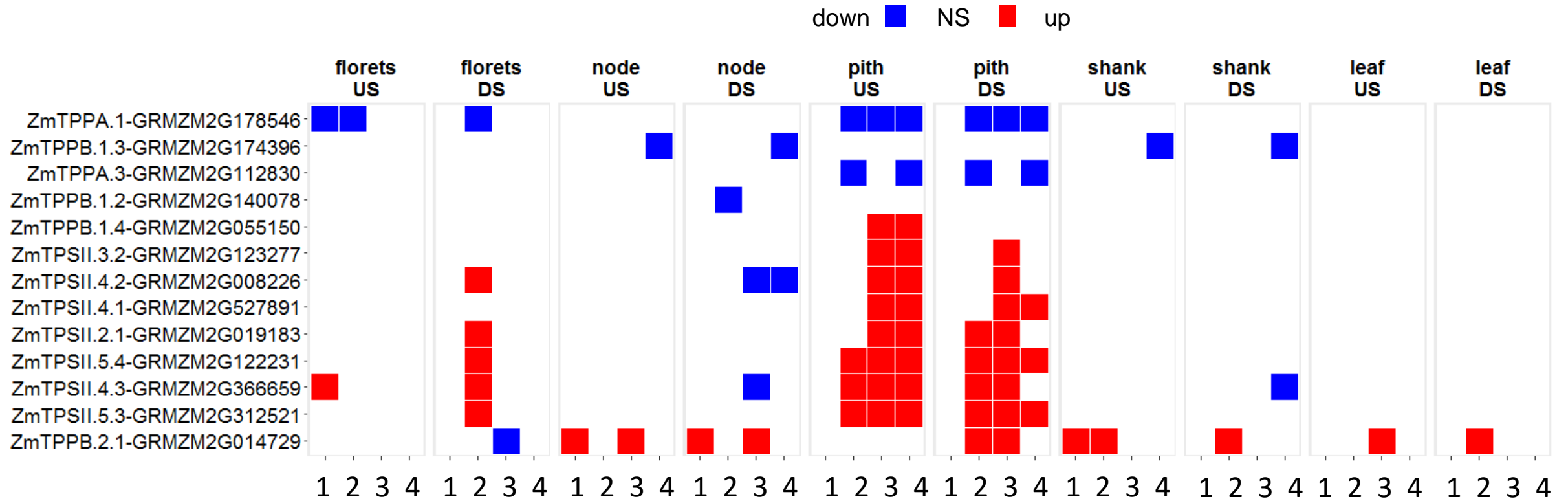
Figure 9. *OSMADS6: TPP1* effect on trehalose metabolism gene expression, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) gene family members evaluated using differential expression analysis. Only genes that changed significantly in both events in the same direction temporally and spatially are reported. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

Figure 10. Heat map showing the effect of *OSMADS6: TPP1* on SWEET genes. Heat maps represent differential expression analysis between wild type and transgenic lines 5217 and 5224. Only genes that changed significantly in both events in the same direction temporally and spatially are reported. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).
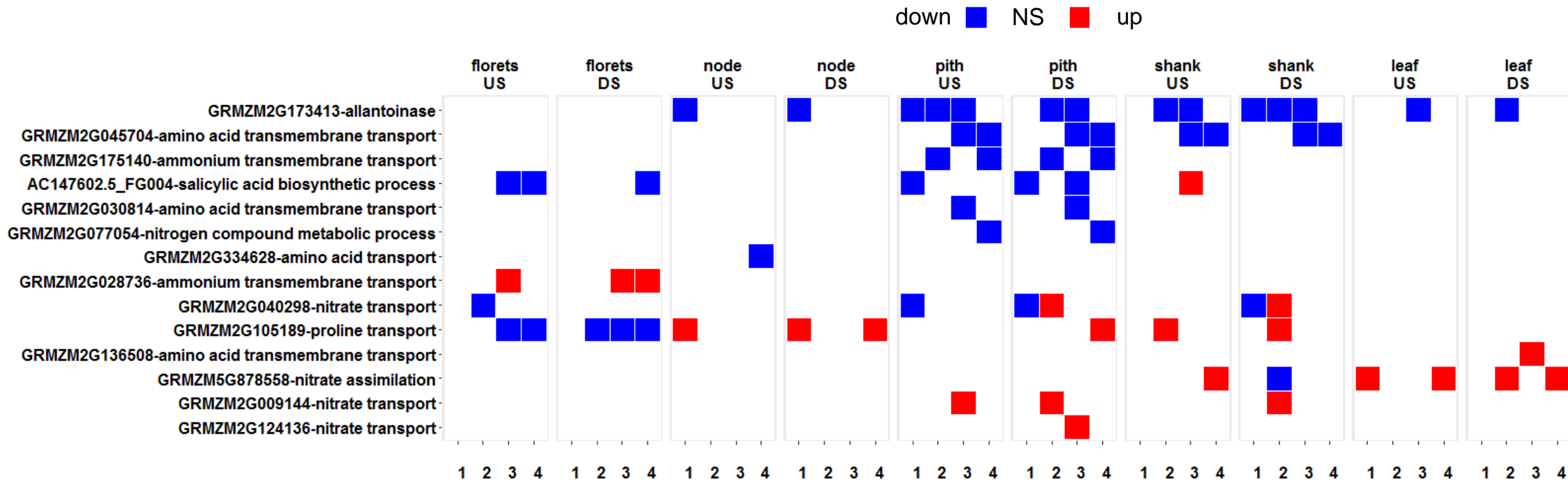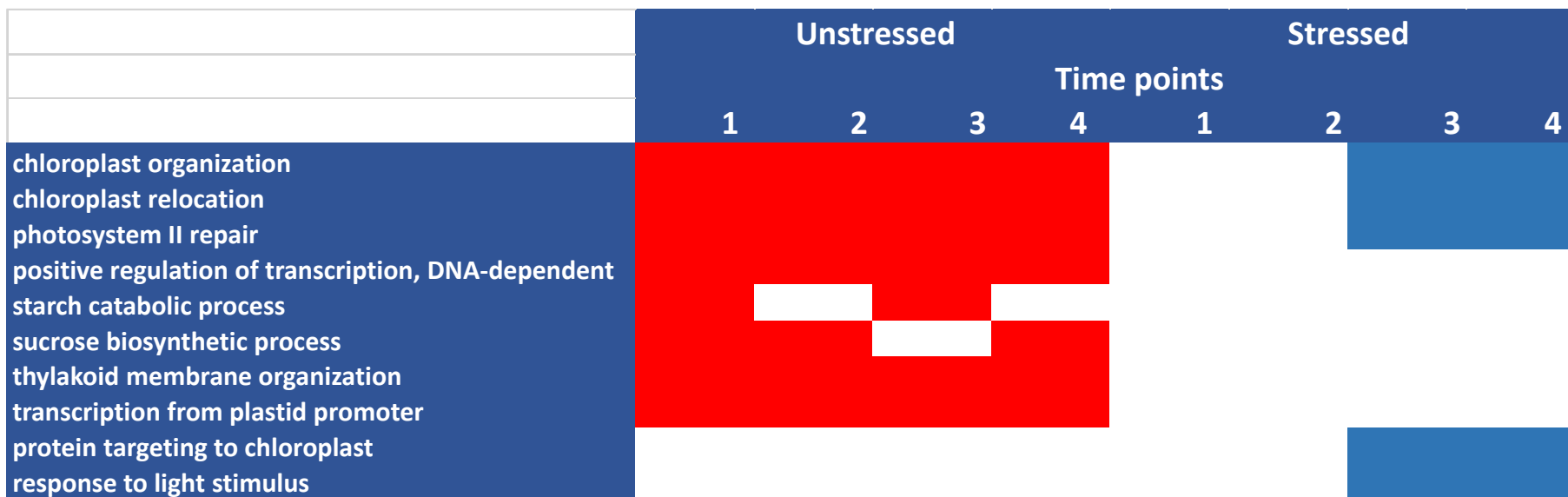
Figure 11. Heat map of differential expression of allantoinase and nitrogen metabolism genes in transgenic lines compared to wild type. Only genes that changed significantly in both events in the same direction are reported. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).
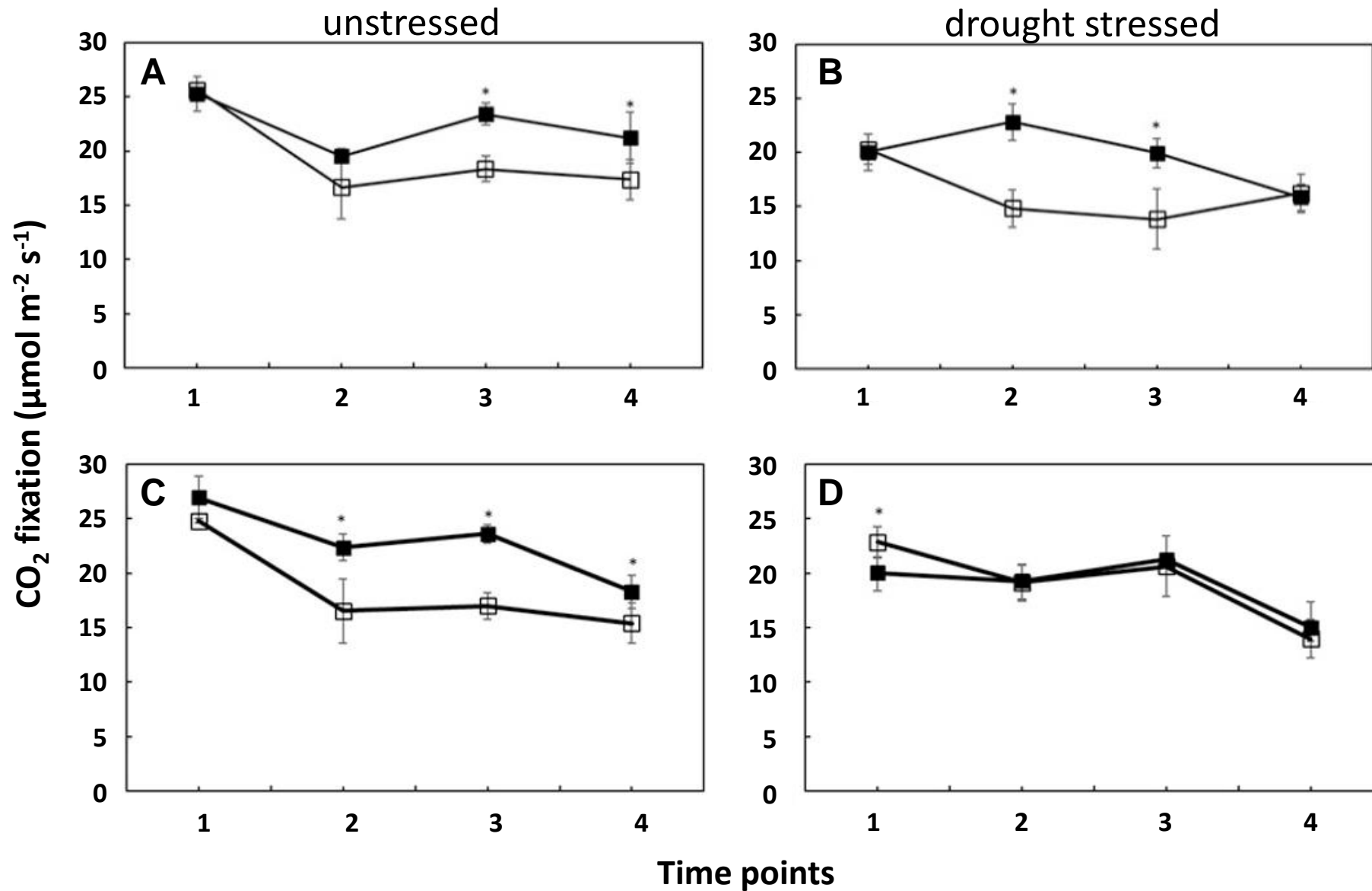
Figure 12. The effect of *OSMADS6: TPP1* on photoassimilation in leaf during reproductive development. The CO_2 uptake rate was measured using an infrared gas analyzer in (A, B) the ear leaf and (C,D) the leaf above the ear during the two week reproductive period (need better context). Unstressed (A, C) and drought stressed (B,D). Wildtype □ and event 5224 ■ plants were evaluated. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4). Data are the mean ± SD (n = 4). * denotes statistical significance at p< 0.05.

GO term	Unstressed				Stressed			
Time points	1	2	3	4	1	2	3	4
chloroplast organization	■	■	■	■		■	■	■
chloroplast relocation	■	■	■	■		■	■	■
photosystem II repair	■	■	■	■		■	■	■
positive regulation of transcription, DNA-dependent	■	■	■	■				
starch catabolic process	■			■				
sucrose biosynthetic process	■	■		■				
thylakoid membrane organization	■	■	■	■				
transcription from plastid promoter	■	■	■	■				
protein targeting to chloroplast						■	■	■
response to light stimulus						■	■	■

Figure 13. *OSMADS6: TPP1* affected genes with gene ontology terms associated with photosynthesis. Transcript profiling data from both the 5217 and 5224 events were compared to wildtype to identify significantly perturbed GO terms. A white cell indicates pathways that were not affected and a dark cell indicates pathways that were significantly affected by the transgene. Only pathways significantly enriched in both events are shown. The time points are 5 days before pollination (1), day of pollination (2), 5 days after pollination (3) and 10 days after pollination (4).

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