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1 **Short title:** Suc phloem loading and retrieval by *ZmSut1*

2

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6

7 **Sucrose transporter *ZmSut1* expression and localization uncover new**
8 **insights into sucrose phloem loading**

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17

18 **One sentence summary:** Maize *Sucrose transporter1* functions to load sucrose into
19 phloem companion cells, restrict its accumulation in the apoplasm, and prevent its loss
20 during long-distance transport.

21

22 **Author Contributions:** R.F.B. participated in the design of the study, conducted the
23 light, epi-fluorescence, and confocal microscopy, performed the CFDA experiments, and
24 drafted the manuscript. K.A.L. performed the qRT-PCR and helped draft the manuscript.
25 N.B. performed genotyping to propagate transgenic events and the quantification of
26 relative signal abundance in the RNA *in situ* hybridizations. M.S. performed genotyping
27 and phenotyping to confirm and propagate transgenic events. Y.B.A., T.S., and D.J.
28 constructed the pZmSut1::RFP and gSUT1-YFP transgenic lines, and A.L. and A.S.
29 provided the PIP2-1-CFP line. D.M.B. conceived of the study, participated in its design
30 and implementation, performed the genetic experiments, and helped draft the manuscript.
31 All authors edited and critically revised the manuscript.

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51

52 **Abstract**

53 Sucrose (Suc) transporters (SUTs) translocate Suc across cellular membranes, and, in
54 eudicots, multiple SUTs are known to function in Suc phloem loading in leaves. In maize
55 (*Zea mays* L.), the *Sucrose transporter1* (*ZmSut1*) gene has been implicated in Suc
56 phloem loading based upon RNA expression in leaves, electrophysiological experiments,
57 and phenotypic analysis of *zmsut1* mutant plants. However, no previous studies have
58 examined the cellular expression of *ZmSut1* RNA or subcellular localization of the
59 ZmSUT1 protein to assess the gene's hypothesized function in Suc phloem loading or to
60 evaluate its potential roles, such as phloem unloading, in non-photosynthetic tissues. To
61 this end, we performed RNA *in situ* hybridization experiments, promoter: reporter gene
62 analyses, and ZmSUT1 localization studies to elucidate the cellular expression pattern of
63 the *ZmSut1* transcript and protein. These data showed *ZmSut1* was expressed in multiple
64 cell types throughout the plant, and indicated it functions in phloem companion cells to
65 load Suc, and also in other cell types to retrieve Suc from the apoplasm to prevent its
66 accumulation and loss to the transpiration stream. Additionally, by comparing a phloem-
67 mobile tracer with *ZmSut1* expression, we determined that developing maize leaves
68 dynamically switch from symplasmic to apoplasmic phloem unloading, reconciling
69 previously conflicting reports, and suggest that *ZmSut1* does not have an apparent
70 function in either unloading process. A model for the dual roles for *ZmSut1* function
71 (phloem loading and apoplasmic recycling), *Sut1* evolution, and its possible use to
72 enhance Suc export from leaves in engineering C₃ grasses for C₄ photosynthesis is
73 discussed.

74

75 **Keywords:** apoplasm, CFDA, maize, phloem, sink, source, Suc, SUT, symplasm,
76 ZmSUT1

77

78 **INTRODUCTION**

79 Plant growth, development, and ultimately crop yield are dependent on the transport of
80 photosynthates from the source (net exporting) leaves to sink (net importing) tissues (e.g.,
81 ears, tassels, stems, roots). In the coming decades, a growing world population (predicted
82 to increase by more than two billion people by 2050) will place increasing pressure on

83 agricultural systems already challenged with the increased temperatures and more erratic
84 precipitation patterns predicted for climate change (Godfray et al., 2010; Rosenzweig et
85 al., 2014). Hence, understanding the transport pathways and genes functioning to control
86 the allocation of carbohydrates in plants will be crucial to improve crop resilience to
87 biotic and abiotic stress and to increase crop productivity (Rennie and Turgeon, 2009;
88 Bihmidine et al., 2013; Lemoine et al., 2013; Braun et al., 2014; Jia et al., 2015; Yadav et
89 al., 2015; Durand et al., 2016).

90

91 To sustain development and growth, photoassimilates must be transported from the
92 leaves through the veins to various sink tissues. In the majority of crop plants, including
93 maize (*Zea mays* L.), sucrose (Suc) is the carbohydrate translocated long-distance from
94 source to sink tissues (Heyser et al., 1978; Ohshima et al., 1990). Suc is synthesized in
95 the mesophyll (M) cells of mature leaves and ultimately enters the phloem tissue within
96 the veins for long-distance transport (Lunn and Furbank, 1999; Slewinski and Braun,
97 2010a). Suc movement from the M cells into the phloem involves a combination of
98 symplasmic and apoplasmic transport (Braun and Slewinski, 2009). In symplasmic
99 transport, Suc moves directly between cells through plasmodesmata (PD), cell-wall pores
100 connecting the cytoplasm of adjacent cells. In apoplasmic transport, Suc is released into
101 the apoplasm (the cell-wall free space) and uptaken into recipient cells (Lalonde et al.,
102 2004; Ayre, 2011; Baker et al., 2012; Chen et al., 2012).

103

104 Suc loading and transport through the phloem primarily occur in different vein types. In
105 maize leaves, three distinct classes of longitudinal veins (lateral, intermediate, and small)
106 function in photoassimilate loading and transport (Russell and Evert, 1985). The majority
107 of Suc phloem loading occurs in the small and intermediate veins, which are collectively
108 termed the minor veins. The Suc is then funneled through small, transversely oriented
109 veins into the lateral veins, which function primarily in long-distance transport from the
110 leaf blade and into other plant regions (Fritz et al., 1983; Fritz et al., 1989).

111

112 Within the phloem, Suc transport occurs in the sieve tube, which is constituted of sieve
113 elements (SE) arranged end-to-end (Evert, 1982). Upon maturation, SE lose their nucleus

114 and most other organelles to form the conducting sieve tube (Esau, 1977), and come to
115 depend on companion cells (CC) for metabolic support and survival (van Bel and
116 Knoblauch, 2000). The transfer of Suc, other metabolites, RNA, and proteins from the
117 CC into the SE occurs symplasmically through the PD connecting them. Because of this
118 dependency, the two cells are referred to as the CC/SE complex.

119

120 The phloem system can be divided into three functionally overlapping domains: the
121 collection, transport, and release phloems (van Bel, 1996). The collection phloem is
122 located in mature source leaf veins and is the site where Suc is loaded into the phloem
123 (Patrick, 2012). The transport phloem connects the collection phloem to the release
124 phloem and is the largest portion of the phloem network in a plant (van Bel, 2003). In the
125 release phloem, Suc is unloaded from the phloem cells into surrounding cells for
126 utilization, storage, or growth (Patrick, 2012). Once Suc enters the collection phloem, it is
127 exported through the transport phloem in the blade and sheath (the leaf base), and then
128 through the stem to distal sink tissues. The mechanism driving this flow of Suc and other
129 solutes through the phloem is the hydrostatic pressure differential generated by the
130 difference in osmotic potentials between the collection and release phloems (Patrick,
131 2012). However, the high concentration of Suc in the sieve tubes relative to the apoplasm
132 poses a thermodynamic challenge to its continued transport. In maize, the Suc
133 concentration has been measured at 0.9–1.4 M in the phloem sieve tube sap (Ohshima et
134 al., 1990; Weiner et al., 1991) and estimated to be 1–3 mM in the leaf apoplasm (Heyser
135 et al., 1978; Lohaus et al., 2000; Lohaus et al., 2001). Thus, Suc is continually lost to the
136 apoplasm by passive leakage across the sieve tube plasma membrane during long-
137 distance transport through the phloem and must be continuously retrieved to maintain the
138 hydrostatic pressure gradient between source and sink tissues (Minchin and Thorpe,
139 1987; Patrick, 2012).

140

141 The other conducting tissue within veins, the xylem, transports water and dissolved
142 minerals from roots to transpiring leaves within the tracheids and vessels (also called
143 elements), which are both dead at maturity (Esau, 1977). In early organ development,
144 vascular tissues are referred to using the prefix “proto” and are termed the protoxylem

145 and protophloem. These tissues are obliterated during organ elongation and growth and
146 are replaced by later-forming metaxylem and metaphloem, which comprise the
147 conducting tissues at maturity (Esau, 1977).

148

149 The different classes of maize veins are distinguished anatomically. Lateral veins contain
150 large metaxylem elements, a protoxylem lacunae (space) produced by the rupturing of the
151 protoxylem elements, and hypodermal sclerenchyma (HS) cells above and below the vein
152 for structural support (Esau, 1977). Intermediate veins contain HS cells on one or both
153 sides of the vein but lack large metaxylem vessels. Small veins lack both HS cells and
154 large metaxylem vessels. Interestingly, ultrastructural studies of the sink-to-source
155 transition in a maize leaf revealed that all veins classes were structurally mature prior to
156 the cessation of phloem unloading (Evert et al., 1996a).

157

158 As a NADP-malic enzyme type of C₄ plant, maize exhibits Kranz anatomy in the leaf
159 blade (Esau, 1977). In particular, two different types of photosynthetic cells
160 concentrically enclose the vein: a single inner layer of bundle-sheath (BS) cells and a
161 single outer layer of M cells (Fig. 2A, B). Both photosynthetic cell types share dense PD
162 connections for facilitating the symplasmic flow of metabolites between them, including
163 Suc (Evert et al., 1977). The BS cells similarly show abundant PD connections with the
164 vascular parenchyma (VP) cells, which are associated with either the xylem (referred to
165 as xylem parenchyma [XP] cells) or phloem (i.e., phloem parenchyma [PP] cells) (Evert
166 et al., 1978). Based on the nearly complete symplasmic isolation of the CC/SE complex
167 from other cell types in the vein, the PP cells are hypothesized to efflux Suc into the
168 apoplasm for subsequent uptake across the plasma membrane into the CC/SE complex
169 (Evert et al., 1978; Baker et al., 2012; Braun, 2012; Chen et al., 2012). In addition to the
170 CC/SE, radioactive labeling studies determined that C¹⁴-Suc is retrieved from the xylem
171 by XP cells, but the transporters responsible for Suc uptake remain unknown (Fritz et al.,
172 1983).

173

174 Multiple classes of transporters involved in Suc flux across cell membranes have been
175 identified, including Suc transporters (SUTs) (Aoki et al., 2003; Lalonde et al., 2004;

176 Sauer, 2007; Kühn and Grof, 2010; Ainsworth and Bush, 2011; Ayre, 2011; Baker et al.,
177 2012; Reinders et al., 2012; Eom et al., 2015; Jung et al., 2015; Bihmidine et al., 2016).
178 However, much remains to be clarified with respect to their particular roles in the phloem
179 loading of Suc in photosynthetic tissues, its long-distance transport, and its unloading in
180 sink tissues, especially in the grasses (Aoki et al., 2003; Braun and Slewinski, 2009;
181 Bihmidine et al., 2013). Based on phylogenetic analysis, the *Sut* genes in plants have
182 been classified into five different groups (Braun and Slewinski, 2009). The group 2
183 (formerly type I) *Sut* genes were the first class of characterized *Sut* genes and are unique
184 to eudicots. Some of these genes show strong expression in mature leaves, and both yeast
185 (*Sacchomyces cerevisiae*) and *Xenopus laevis* oocyte heterologous expression studies
186 of various SUT proteins demonstrated that they possess Suc transporter activity
187 (Riesmeier et al., 1992; Aoki et al., 2003; Chandran et al., 2003; Carpaneto et al., 2005;
188 Sivitz et al., 2005; Reinders et al., 2006; Sun et al., 2010). Additional mutational analyses
189 and RNA suppression experiments supported a role for the group 2 *Sut* genes in Suc
190 loading into the phloem (Riesmeier et al., 1994; Bürkle et al., 1998; Gottwald et al.,
191 2000; Hackel et al., 2006; Srivastava et al., 2008). Recent experiments have also found
192 that the *AtSUC2* gene in *Arabidopsis thaliana*, which was known to function in phloem
193 loading, also performs Suc retrieval in the transport phloem (Srivastava et al., 2008;
194 Gould et al., 2012).

195

196 Group 2 *Sut* genes are absent from monocot genomes; hence, the group 1 *Sut* genes,
197 which are unique to the monocots, have been proposed to function in Suc phloem loading
198 in leaves (Aoki et al., 2003; Sauer, 2007; Braun and Slewinski, 2009; Kühn and Grof,
199 2010). Based on their broad expression in both source and sink tissues, some group 1 *Sut*
200 genes have also been hypothesized to function in Suc phloem unloading in sink tissues
201 and in the retrieval of leaked Suc along the phloem transport route, as shown by studies
202 in rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize, and sugarcane (*Saccharum*
203 *officinarum*). In rice, OsSUT1 has been localized to the SE and CC in the veins of the
204 mature leaf, stems, pedicel, and base of the filling grain (Scofield et al., 2007). However,
205 despite expression in the CC/SE complex in the leaf, no effect on photosynthesis or
206 carbohydrate contents was observed in the leaves of rice lines with strong antisense

207 repression of the *OsSUT1* RNA (Ishimaru et al., 2001; Scofield et al., 2002). Further
208 analysis of mutant plants homozygous for a null *OsSUT1* allele produced by a *Tos17*
209 retrotransposon insertion confirmed the absence of a phenotype in the vegetative leaves
210 (Eom et al., 2012). These findings were interpreted as support that *OsSUT1* does not play
211 a major role in phloem loading in the mature rice leaf (Braun et al., 2014). By contrast,
212 grain filling and germination were impaired in the RNA-suppression lines (Ishimaru et
213 al., 2001; Scofield et al., 2002). Moreover, in an expression analysis of an *OsSUT1*
214 promoter:: β -glucuronidase (GUS) transgene, the XP cells and the cells at the border of
215 the phloem/xylem interface showed slight, sporadic expression of the transgene in the
216 minor veins in the mature leaf under normal physiological conditions, and increased
217 expression after aphid feeding (Scofield et al., 2007; Ibraheem et al., 2014). Based on
218 these studies, *OsSUT1* has been proposed to function in Suc retrieval along the transport
219 phloem and from the xylem upon insect herbivory (e.g., aphid feeding) (Scofield et al.,
220 2007; Eom et al., 2012; Braun et al., 2014; Ibraheem et al., 2014). Similarly, the
221 expression of wheat *TaSUT1* RNA and the localization of its encoded protein in leaves
222 were restricted to the CC and SE, respectively (Aoki et al., 2004). Intriguingly, sugarcane
223 *ShSUT1* was not localized to the CC/SE complex in the leaf or stem, but instead to the
224 XP and PP cells in lateral and intermediate veins of the leaf and in non-conducting cells
225 in the stem, indicating that like *OsSUT1*, *ShSUT1* also does not function in phloem
226 loading, but may function to retrieve Suc lost to the apoplasm (Rae et al., 2005). These
227 data indicate that the roles of group 1 *Sut* genes remain to be resolved and that their
228 functions may vary between the grasses (Braun and Slewinski, 2009; Braun et al., 2014;
229 Bihmidine et al., 2015). Moreover, these results leave in doubt whether any group 1 *Sut*
230 gene functions in phloem loading in grasses.

231

232 Based on homology with rice *OsSut1*, the first *Sut* gene cloned from maize was *ZmSut1*
233 (Aoki et al., 1999). *ZmSut1* was found to show high expression levels and diurnal cycling
234 of its transcript in mature leaves and to be expressed in various sink tissues. Based on this
235 expression profile, *ZmSut1* was proposed to function in the phloem loading of Suc in
236 mature leaves and potentially to perform the phloem unloading of Suc into organs such as
237 the pedicel. Subsequent oocyte expression studies supported the ability of *ZmSUT1* to

238 move Suc across a membrane in a reversible manner based on the direction of the Suc
239 gradient across the membrane, the pH gradient, and the membrane potential (Carpaneto et
240 al., 2005; Geiger, 2011). From these studies, the authors proposed that ZmSUT1 could
241 function to unload Suc from the phloem in sink tissues. Consistent with a role in phloem
242 loading, phenotypic characterization of *zmsut1* null mutants revealed stunted plant
243 growth, frequent failure to achieve reproductive maturity, chlorotic leaves that
244 accumulated excess levels of starch and soluble sugars (e.g., Suc, Glc, and Fru), and
245 impaired transport of radioactively labelled Suc out of the leaves (Slewinski et al., 2009;
246 Rotsch et al., 2015). Another intriguing phenotype of *zmsut1* mutant plants was the
247 secretion of droplets with high Suc concentrations from the hydathodes (Supp. Fig. S1)
248 (Slewinski et al., 2010). Because no Suc was detectable in the wild-type guttation fluid,
249 this finding indicated a high level of Suc was present in the apoplasm (which is
250 contiguous with the xylem transpiration stream) of *zmsut1* mutants, and supported a role
251 for *ZmSut1* in phloem loading. In addressing this potential role, one previous study
252 evaluated *ZmSut1* expression in vein-enriched vs. non-vein tissues dissected from the
253 coleoptile, the protective sheath enclosing the germinating shoot, and found higher
254 expression in the vein-enriched tissue (Bauer et al., 2000). However, to our knowledge,
255 no previous studies have examined the cellular and subcellular expression of *ZmSut1* and
256 the ZmSUT1 protein, respectively, to evaluate this proposed role in the phloem loading
257 of Suc in the leaf blade. Further, only limited studies have investigated the potential role
258 of *ZmSut1* in non-photosynthetic tissues.

259

260 Although apoplasmic phloem loading in maize is physiologically and anatomically well
261 established (Evert et al., 1978; Heyser et al., 1978; Slewinski et al., 2009; Slewinski et
262 al., 2012), the exact role of *ZmSut1* in this process is not well defined. Specifically, does
263 ZmSUT1 mediate Suc phloem loading? If so, several predictions based on the model for
264 apoplasmic phloem loading are that *ZmSut1* will be expressed in the CC/SE complex of
265 mature leaves and that ZmSUT1 will be localized to the plasma membrane. To
266 investigate these predictions, we used RNA *in situ* hybridization to examine *ZmSut1*
267 expression at the cellular level in mature leaf tissues. These results were extended to
268 additional tissues within the plant by investigating the cellular expression pattern of the

269 native *ZmSut1* promoter driving a transcriptional reporter gene encoding the monomeric
270 red fluorescent protein (RFP). Additionally, the subcellular localization of the ZmSUT1
271 protein translationally tagged with the yellow fluorescent protein (YFP) was determined.
272 Coupled with the previous phenotypic characterization of *zmsut1* mutants, these findings
273 inform our understanding of the function of *ZmSut1* in the collection and transport
274 phloems.

275

276 In contrast to our understanding of Suc phloem loading in mature leaves, the phloem
277 unloading pathway in maize is less resolved and has been suggested to be apoplasmic in
278 leaves (Evert and Russin, 1993) and symplasmic in roots (Giaquinta et al., 1983;
279 Warmbrodt, 1985; Hukin et al., 2002; Ma et al., 2009). However, Haupt et al. (2001)
280 determined that the phloem unloading pathway in developing leaves in the related grass
281 barley (*Hordeum vulgare*) is symplasmic rather than apoplasmic, calling into question the
282 conclusions based upon ultrastructural studies that both barley and maize sink leaves use
283 apoplasmic phloem unloading (Evert and Russin, 1993; Evert et al., 1996b). To better
284 resolve the pathway utilized for phloem unloading in maize immature leaves and roots,
285 we analyzed transport and phloem unloading of the phloem-mobile tracer
286 carboxyfluorescein (CF) in these tissues, and investigated whether *ZmSut1* has any role in
287 Suc efflux as proposed by Carpaneto et al. (2005). Interestingly, our data reconcile the
288 previously contradictory results and provide a framework to understand *ZmSut1* function
289 in the transport and release phloems. Finally, our data also suggest an enhanced role of
290 apoplasmic Suc retrieval in nonconducting vascular cells in leaves in the context of C₄
291 photosynthesis, providing insights as to how *Sut* gene expression could be tailored for
292 engineering C₄ photosynthesis and carbon transport in C₃ plants such as rice.

293

294 RESULTS

295 ***ZmSut1* cellular expression is consistent with phloem loading but indicates** 296 **additional functions**

297 Previous analysis of *ZmSut1* expression in the mature third leaf blades from 2-week-old
298 greenhouse-grown plants found the transcript levels peaked at the end of the d and
299 decreased during the night to low levels, with expression increasing again the following

300 afternoon (Aoki et al., 1999). An evaluation of the expression pattern in immature leaf
301 tissues was limited to examining the apical-to-basal expression pattern in expanding leaf
302 blades, which showed a gradient of very high expression at the tip to very low expression
303 at the base (Aoki et al., 1999). To evaluate whether *ZmSut1* expression in developing leaf
304 tissue also followed a diurnal pattern as reported for mature juvenile leaves, we
305 performed quantitative reverse-transcription polymerase chain reaction (qRT-PCR)
306 experiments on both mature adult (leaf 11) and immature adult (leaf 17) leaves of 6-
307 week-old field-grown B73 plants for a period of 48 hrs during the d/night cycle (Fig. 1).
308 In contrast to previous results, *ZmSut1* transcript abundance showed no pattern of
309 detectable fluctuation in mature leaves over the course of the 24-h cycle, and similarly
310 showed no diurnal pattern in immature leaf tissues. Although *ZmSut1* mRNA transcript
311 levels tended to be slightly higher in immature than mature adult leaves, the increased
312 expression was not significant. These differences in *ZmSut1* leaf expression prompted us
313 to further examine its expression.

314

315 Based on previous work, it was anticipated *ZmSut1* would be expressed in the phloem of
316 mature leaf blades because of its postulated role in Suc loading. To determine the cellular
317 expression pattern of *ZmSut1* in mature leaf blade tissue, we performed RNA *in situ*
318 hybridization. As evidenced by the blue precipitate indicative of gene expression, *ZmSut1*
319 expression was indeed observed in the CC in both the minor and lateral veins (Fig. 2C-
320 H). By contrast, *ZmSut1* expression was absent from the enucleate SE. Interestingly,
321 *ZmSut1* expression was also strongly detected in the BS, PP, and XP cells of both lateral
322 and minor veins (Fig. 2C, D, F). In the minor veins, *ZmSut1* expression was consistently
323 observed in the VP cells and CC. Signal was sporadically seen in the BS cells and often
324 observed in only a subset of them around the vein (Fig. 2C, D). In minor veins, the VP
325 cells near the xylem (i.e., XP cells) are typically larger relative to the phloem-associated
326 VP cells. These large XP cells usually exhibited the strongest *ZmSut1* expression
327 compared to the other VP cells and cell types. In lateral veins, *ZmSut1* was strongly
328 expressed in the XP cells, particularly near the metaxylem elements and protoxylem
329 lacunae, and in the CC and sporadically in the BS cells (Fig. 2F). As a negative control,
330 we analyzed *ZmSut1* expression in *zmsut1* mutants, which result from a *Ds* transposable

331 element insertion into the 5' untranslated region of the gene that greatly reduces *ZmSut1*
332 RNA abundance (Slewinski et al., 2010) (Fig. 2G). These experiments showed a greatly
333 reduced signal in the same subset of cells as in the wild-type B73 leaf. We used qRT-
334 PCR to determine whether residual expression could account for the reduced signal
335 observed in the mutant plants (Supp. Fig. S2). However, expression in the *zmsut1* mutant
336 was less than 6.5% that of the wild type, suggesting the majority of the signal had a
337 different underlying cause. Some of this signal may be due to background staining, as
338 control sections lacking the *ZmSut1* probe displayed blue staining in the epidermis and to
339 a limited extent in the xylem and HS cells (Fig. 2E, H). Overall, these findings
340 demonstrate that *ZmSut1* is expressed in the CC, where it likely functions to load Suc into
341 the phloem, as well as broadly in all other cells enclosed within the vascular bundle, with
342 the exception of the enucleate SE and the xylem vessels, which are dead at maturity.

343

344 From the RNA *in situ* hybridization data, *ZmSut1* expression in the CC unexpectedly
345 appeared to comprise only a small portion of the total signal. To evaluate the relative
346 abundance of *ZmSut1* RNA expression in the CC/SE compared to the remainder of cells
347 within the vein and the BS cells, the percentage of blue signal in each cell type in each
348 vein class was quantified with Image J (Table 1). Based on their position within the vein
349 and their size, the cells were classified as CC/SE, VP (XP + PP), or BS cells. For these
350 measurements, we focused our attention on the veins; any signal detected in the xylem
351 elements, HS cells, and epidermis was excluded as non-specific. We note that these data
352 are only a rough approximate of the signal abundance in each cell type and do not
353 adequately account for differences in cell size, vacuole size, signal intensity, or
354 representation of cell types in the sections. However, they do provide an estimate of the
355 relative amount of signal within the CC/SE compared to other cell types. In the lateral
356 veins, the *ZmSut1* expression in the CC/SE was ~14% of the blue signal, the VP cells
357 accounted for nearly 81% of the signal, and the BS cells ~5% (Table 1). By contrast, in
358 the minor veins, the BS cells contained ~27% of the blue signal, while the CC
359 represented only ~15% of the signal, with the VP cells accounting for ~55-60%. The
360 principal differences in the relative signal abundance between the lateral and minor vein
361 classes appeared to be due to the increased percentage of signal contained in BS cells,

362 and the corresponding decreased abundance of signal within the VP cells. Overall, there
363 was little difference in the contribution of *ZmSut1* signal between the CC of different vein
364 classes, and the *ZmSut1* expression pattern was highly similar between the intermediate
365 and smallest veins. There was approximately six-to-eleven-fold more *ZmSut1* signal in
366 the VP and BS cells compared to the CC across all vein classes (Table 1). These data
367 suggest that the majority of *ZmSut1* RNA is expressed in vascular-associated cells other
368 than the CC in longitudinal veins.

369

370 **A transcriptional reporter gene faithfully reveals *ZmSut1* cellular expression** 371 **throughout the plant**

372 Because seven *Sut* genes are present in the maize genome (Braun et al., 2014), some of
373 the faint blue signal detected in the *zmsut1* mutant (Fig. 2G) and wild-type (Fig. 2C, D, F)
374 leaf tissue in the RNA *in situ* hybridization might be due to expression of a closely
375 related *Sut* gene, such as *ZmSut7*. To specifically examine the expression of *ZmSut1*, we
376 analyzed a transgenic transcriptional reporter line. Approximately 2 kb of promoter
377 upstream from the translation start signal was cloned in front of a version of RFP targeted
378 to the endoplasmic reticulum (pZmSut1::RFP_{ER}), and stably transformed into maize. The
379 expression of the pZmSut1::RFP_{ER} reporter gene in the mature leaf blade (Fig. 3)
380 mirrored the expression shown by RNA *in situ* hybridization (Fig. 2). In particular, RFP
381 expression was strong in the CC, PP, and XP cells in the lateral and minor veins in both
382 transverse and paradermal tissue sections (Fig. 3A, C, D, F, H). Additionally, RFP
383 expression varied in intensity between BS cells within the same vein for each vein class.
384 Non-transgenic control sibling plants had virtually no RFP signal (Fig. 3B, E, G, I).
385 Identical results were observed in two additional independent transformation events of
386 the pZmSut1::RFP_{ER} reporter gene. These results indicated that the RFP signal was the
387 same as the expression observed by RNA *in situ* hybridization, and hence, that the
388 pZmSut1::RFP_{ER} transcriptional reporter gene could be used to reliably monitor *ZmSut1*
389 expression.

390

391 To determine which cells expressed *ZmSut1* in immature leaves, we used the
392 pZmSut1::RFP_{ER} transgene to examine expression within the longitudinal (proximal-

393 distal) axis of the leaf (Supp. Fig. S3 and Fig. 4). We selected a leaf undergoing the sink-
394 to-source transition, with the tip emerging out of the whorl into the light, to be able to
395 compare pZmSut1::RFP expression with the characterized anatomical, developmental,
396 and physiological changes that occur during this transition (Evert et al., 1996a). In
397 developing leaf blade tissue in which metaxylem and metaphloem had fully formed in the
398 lateral veins, pZmSut1::RFP expression was evident throughout the leaf blade,
399 including the BS, M, and epidermal cells, but was strongest in the CC, XP, and PP cells
400 within all vein classes (Fig. 4C, D). In leaves of non-transgenic control plants,
401 autofluorescent red signal was visible only from the HS cell walls (Fig. 4A, B), showing
402 that under these microscope and camera settings, none of the observed red signal in the
403 transgenic leaves was due to chlorophyll autofluorescence. In younger, more proximal
404 developing leaf blade and sheath tissue, in which only protoxylem and protophloem were
405 evident within the lateral veins, strong RFP signal was observed in the XP cells bounding
406 the protoxylem elements and in the protophloem cells (Fig. 4E-H, M, N). However,
407 expression was largely excluded from the developing procambium of developing leaves
408 near the meristem, with punctate signal only visible in initiating lateral veins (Fig. 4I, J).
409 Thus, in the youngest cells at the base of the leaf, pZmSut1::RFP expression was
410 largely confined to initiating lateral veins, and expression expanded into non-vascular
411 cells as leaf maturation progressed.

412

413 *ZmSut1* exhibits an increasing base-to-tip gradient of expression (Supp. Fig. S3) (Aoki et
414 al., 1999; Li et al., 2010) that mirrors and is potentially linked to the sink-to-source
415 transition within a leaf. However, it is possible the expression pattern instead reflects the
416 leaf developmental (age) and/or the light-regulated genetic programs. To test whether
417 light exposure influenced pZmSut1::RFP expression, we germinated seedlings in the
418 dark. After 8 d, leaf 1 of the etiolated seedlings had emerged from the coleoptile, and a
419 small tissue segment located on one side of the midrib was harvested for microscopic
420 analysis. We observed a similar pattern of pZmSut1::RFP expression as in developing
421 leaves within the whorl (cf. Fig. 5A, C with Fig. 4C, D). The etiolated plants were
422 subsequently exposed to white fluorescent lights in the lab for 48 hrs to induce
423 chlorophyll synthesis and transferred into the greenhouse with high light. After 5 d in the

424 greenhouse, the other half of leaf 1, which was dark green and fully mature, was analyzed
425 (Fig. 5B, D). We observed strong expression of pZmSut1::RFPper within the CC, XP, and
426 PP cells within the veins in the green tissue relative to the etiolated tissue, supporting an
427 induction of pZmSut1::RFPper expression by light, age, and/or physiological maturity as
428 source tissue.

429

430 To further dissect which of these factors might influence the pZmSut1::RFPper expression
431 pattern, we backcrossed the pZmSut1::RFPper reporter gene into the *striate2* (*sr2*) mutant,
432 which displays a variegation pattern of longitudinal white and green leaf stripes (Huang
433 and Braun, 2010). If age or light influenced pZmSut1::RFPper expression, we reasoned
434 expression would be comparable between neighboring green and white mature leaf
435 tissues. Alternatively, if the sink-to-source transition influenced expression, we should
436 see differences between the white (sink) and adjacent green (source) regions, since both
437 regions experience similar light exposure. In *sr2* mutant plants carrying pZmSut1::RFPper,
438 we observed strong up-regulation of pZmSut1::RFPper in the XP and PP in green tissue
439 compared to white tissue, indicating pZmSut1::RFPper is upregulated in source tissues
440 (Fig. 6A, C, E). Non-transgenic *sr2* mutant leaves at green-white borders showed no RFP
441 signal (Fig. 6B, D, F). Additionally, under these microscope and camera settings, no
442 chlorophyll autofluorescence was detected using the RFP filter cube (cf. Fig. 6D, F),
443 indicating the red signal in Fig. 6C resulted from RFP. Note the apparent difference in
444 RFP expression in the M cells between white and green tissues in pZmSut1::RFPper; *sr2*
445 leaves (Fig. 6C) does not result from differences in gene expression, but appears to be
446 due to the chloroplasts masking the underlying RFP signal in green tissue. Moreover, it is
447 worth noting the strong upregulation of pZmSut1::RFPper expression in the veins in
448 source tissues as compared to sink tissues decreased the relative apparent signal in the M
449 cells. Hence, by controlling for leaf age and light exposure, we determined
450 pZmSut1::RFPper expression was enhanced in PP and XP cells in source compared with
451 sink leaf tissue.

452

453 In addition to developing leaves, Aoki et al. (1999) reported *ZmSut1* was expressed in
454 stems (culms), developing tassels (flowers and rachis-branches), and ears (pedicels), but

455 not in roots. We thus examined pZmSut1::RFP_{er} expression in these and other sink
456 tissues to determine the cell-specific expression pattern. For immature stem internode
457 tissue, we observed strong RFP expression in veins and expanding storage parenchyma
458 cells (Fig. 7A, B). However, the signal was strongest in the XP cells surrounding the
459 developing protoxylem and in the protophloem cells. In mature stem internodes, the
460 signal was strongest in the mature veins, with weaker signal in the storage parenchyma
461 cells (Fig. 7C). No signal was visible in non-transgenic controls (Fig. 7D). Closer
462 inspection of mature stem veins with confocal microscopy showed strong
463 pZmSut1::RFP_{er} expression in the CC, XP, and PP cells (Fig. 7E-G). The red signal in
464 the xylem elements and cells surrounding the vein was due to autofluorescence, as
465 evidenced by a non-transgenic control section (Fig. 7H-J).

466

467 In the shoot apical meristem, the rib meristem displayed limited and variable expression
468 of the transgene, while the central and peripheral zones had absent or only very minimal
469 RFP signal (Fig. 8A). However, the stem immediately subtending the meristem exhibited
470 the strongest expression of pZmSut1::RFP_{er} detected in the plant, which is indicated by
471 the saturated orange-yellow signal in Figure 8A. Examination of the developing tassels
472 and ears showed RFP expression was absent from the inflorescence and axillary
473 meristems but was present in the developing tassel rachis and developing cob (Fig. 8C-
474 F). Transverse sections through the ear demonstrated expression in the developing veins
475 leading to the ovules (Fig. 8G-L). In the maturing tassel, expression was strong in the
476 veins (Fig. 8M), including the vein between the anther locules in the male flower (Fig.
477 8N).

478

479 Using northern blot assays, Aoki et al. (1999) did not detect *ZmSut1* expression in roots.
480 By contrast, we observed strong expression in both developing and mature roots (Fig.
481 8O-V). In immature roots, pZmSut1::RFP_{er} expression was initially present in the
482 developing protophloem and protoxylem (Fig. 8O). As development proceeded, its
483 expression was maintained in the phloem, but became reduced in maturing xylem
484 elements undergoing programmed cell death (compare Fig. 8Q and S). In mature root
485 tissue, the phloem showed the strongest expression, with weaker signal in other cell types

486 (Fig. 8U). We observed no RFP signal in any of these tissues in non-transgenic control
487 plants.

488

489 **pZmSut1::RFP** expression is not correlated with the phloem unloading zone

490 Biochemical studies demonstrated that ZmSUT1 was capable of mediating Suc import
491 into cells, and with the appropriate Suc concentration gradient, pH gradient, and electrical
492 potential across the plasma membrane, it could also export Suc from cells (Carpaneto et
493 al., 2005). Therefore, it was postulated ZmSUT1 could function to efflux Suc from the
494 phloem into sink tissues. The strong expression of the pZmSut1::RFP transgene in the
495 phloem of roots (Fig. 8O-V) also suggested it might be involved in phloem unloading.
496 However, previous physiological and anatomical studies indicated that Suc was unloaded
497 symplasmically from the root phloem, suggesting ZmSUT1 might not be involved in
498 phloem unloading (Giaquinta et al., 1983; Warmbrodt, 1985; Hukin et al., 2002; Ma et
499 al., 2009). To assess whether potential apoplasmic unloading via ZmSUT1 overlaps with
500 potential symplasmic unloading via plasmodesmata in the unloading zone, we compared
501 pZmSut1::RFP expression with the symplasmic tracer CF. Carboxyfluorescein
502 diacetate (CFDA) is a membrane-permeable non-fluorescent dye that is converted to the
503 membrane-impermeable, fluorescent form (CF) inside cells to trace solute movement in
504 the phloem (Grignon et al., 1989; Ma et al., 2009; Bihmidine et al., 2015). We fed CFDA
505 to physiologically mature source leaves of developing pZmSut1::RFP transgenic plants,
506 and examined the overlap between RFP and CF signals in root tips. CF was translocated
507 through the phloem and symplasmically unloaded into the pith and cortex cells basal
508 (toward the shoot) to the root differentiation zone (Fig. 9A, B, E, F). No CF signal was
509 detected in the root cap, root meristem, or differentiation zone. pZmSut1::RFP was
510 expressed strongly in the phloem and xylem and to a lesser extent in the surrounding
511 cortical cells and pith (Fig. 9C, G). pZmSut1::RFP expression was observed both
512 basally and apically (toward the root tip) relative to the root phloem unloading zone
513 marked by CF movement into the cortical cells (Fig. 9D, H). Importantly, the region of
514 CF symplasmic unloading from the phloem and pZmSUT1::RFP expression were
515 independent, indicating pZmSut1::RFP expression overlapped with, but was not
516 restricted to, the phloem unloading zone (Fig. 9D, H).

517

518 We similarly investigated CF phloem unloading within the developing sink leaves of
519 pZmSut1::RFP plants. Interestingly, we observed different phloem unloading pathways
520 being utilized in different portions of the same developing leaf (Fig. 10). In enclosed
521 developing sink leaf tissues located approximately half way between the top and base of
522 the fed leaf sheath, we consistently observed that CF was confined to the phloem,
523 indicating that the phloem was symplasmically isolated from surrounding cells (Fig.
524 10C). Of note, this region represents etiolated sink tissue completely enveloped within
525 the whorl, and is less than one-third of the length it will reach before emerging from the
526 whorl, indicating it must import carbon for cell expansion (Evert et al., 1996a). However,
527 at approximately 25% of the distance above the sheath base, within the same developing
528 sink leaf, we observed CF moving symplasmically out of the phloem into adjacent cells
529 (Fig. 10D). These data indicate that maize sink leaves undergo a transition from using
530 symplasmic phloem unloading to apoplastic phloem unloading substantially prior to the
531 sink-to-source transition when leaves emerge from the whorl. pZmSut1::RFP was
532 expressed strongly in the veins and surrounding cells in both regions (Fig. 10E, F).
533 Collectively, these results suggest that pZmSut1::RFP expression within sink tissue is
534 not correlated with the Suc phloem unloading mechanism in root tips, and is also
535 independent of both symplasmic and apoplastic Suc phloem unloading in developing
536 sink leaves.

537

538 **ZmSUT1 localizes to the plasma membrane**

539 A prediction for ZmSUT1 functioning to transport Suc into cells from the apoplasm is its
540 localization to the plasma membrane. To test this hypothesis, we constructed a
541 translational fusion protein of YFP attached to the C-terminus of ZmSUT1 under control
542 of the endogenous genomic regulatory sequences, including the native promoter, all
543 exons and introns, and both the 5' and 3' untranslated regions (referred to as gSUT1-
544 YFP). To assess if the fusion protein was functional, we examined if the transgene
545 complemented the *zmsut1* mutant phenotype. To this end, we twice backcrossed plants
546 carrying gSUT1-YFP to heterozygous *Sut1/sut1* plants to generate families segregating
547 for wild-type and *sut1* mutant plants and the presence or absence of the transgene. All

548 plants were genotyped for *ZmSut1* and the transgene. As previously reported,
549 homozygous *zmsut1* mutant plants were stunted and did not complete their lifecycle in
550 comparison to wild-type plants (Fig. 11) (Slewinski et al., 2009; Slewinski et al., 2010).
551 In contrast, *zmsut1* mutant plants carrying the gSUT1-YFP transgene flowered, set seed,
552 and completed their lifecycle, indicating the transgene was able to restore *ZmSut1*
553 function (Fig. 11; Supp. Table S1). However, the *zmsut1* mutant plants carrying one copy
554 of the transgene attained only ~75% of the height of their wild-type sibling plants,
555 suggesting the transgene largely, but incompletely, complemented the *zmsut1* mutant
556 phenotype. No difference in plant growth or flowering was observed in wild-type plants
557 with or without the gSUT1-YFP transgene. Virtually identical results were observed in
558 two additional independent transformation events of the gSUT1-YFP translational fusion
559 (Supp. Table S1). We conclude that the YFP fusion to the C-terminus of ZmSUT1
560 maintained its biochemical function, albeit partially, and that the gSUT1-YFP transgene
561 was expressed in the correct cells and at the correct time to provide ZmSUT1 function.

562

563 To investigate whether the gSUT1-YFP translational fusion reporter gene exhibited the
564 same expression pattern as the endogenous *ZmSut1* RNA, we crossed plants harboring
565 this construct to plants carrying the pZmSut1::RFPper transcriptional reporter gene, which
566 recapitulated the *ZmSut1* expression detected by RNA *in situ* hybridization studies, to
567 generate doubly labeled plants. As seen in the minor vein of a mature leaf blade, the
568 gSUT1-YFP construct showed the same cellular expression pattern as the
569 pZmSut1::RFPper transgene (Supp. Fig. S4). Non-transgenic control plants displayed no
570 YFP signal in the veins, with only weak autofluorescence detected in the M cells (Supp.
571 Fig. S4D). Examining expression in a lateral vein of a mature leaf of a gSUT1-YFP
572 transgenic plant, we observed YFP expression in the CC, PP, XP, and BS cells, but not in
573 the SE (Fig. 12A-C), a pattern identical to that of the RNA *in situ* hybridization (Fig. 2)
574 and transcriptional reporter (Fig. 3). Collectively, these data indicate that the gSUT1-YFP
575 construct largely complemented the *zmsut1* mutation and was expressed similarly to the
576 endogenous gene; therefore, we used it for protein localization studies.

577

578 Examination of the BS cells in a paradermal section of the mature leaf blade of a gSUT1-
579 YFP transgenic plant revealed ZmSUT1 was present at the cell periphery, adjacent to the
580 cell wall, and therefore likely localized to the plasma membrane (Fig. 13A-C). To
581 evaluate this possibility, we performed co-localization studies with a known plasma
582 membrane-localized protein, the aquaporin PIP2-1 (Zelazny et al., 2007; Mohanty et al.,
583 2009) translationally fused to the cyan fluorescent protein (PIP2-1-CFP). Co-expression
584 of both transgenes in doubly labelled plants confirmed gSUT1-YFP localized to the
585 plasma membrane (Fig. 13D-F). Like PIP2-1-CFP, gSUT1-YFP localization was seen
586 throughout the plasma membrane. However, occasional punctate localization of both
587 gSUT1-YFP and PIP2-1-CFP in the plasma membrane was observed, which we suggest
588 to be PD. To provide further support for this localization pattern, leaves were
589 plasmolysed by using a concentrated NaCl solution. Prior to plasmolysis, the YFP signal
590 was localized at the cell periphery (Fig. 13G). After plasmolysis, multiple YFP-labeled
591 Hechtian strands were observed connecting the plasmolyzed plasma membrane to the cell
592 wall (Fig. 13H) (Lang-Pauluzzi, 2000). Non-transgenic control plants showed only weak
593 background autofluorescence (Fig. 13I). Because the plasma membrane is anchored at the
594 PD, the plasmolysis results provided additional evidence for the plasma-membrane
595 localization of the ZmSUT1-YFP protein.

596

597 **DISCUSSION**

598 Previous research suggested *ZmSut1* plays a role in phloem loading and potentially in
599 phloem unloading. However, since the orthologous sugarcane and rice *Sut1* genes have
600 no apparent function in phloem loading, the cell-type-specific expression and possible
601 functions of the maize *Sut1* gene were uncertain. To address its role in both source and
602 sink tissues, we used a combination of approaches to investigate the cellular expression
603 of the *ZmSut1* transcript and protein. We determined that *ZmSut1* was expressed in all
604 vein classes and phloem domains (e.g., collection phloem) throughout the plant.
605 Additionally, these experiments yielded several unexpected results, including 1) a lack of
606 diurnal cycling of the transcript in adult leaves, 2) the majority of *ZmSut1* expression in
607 the source leaf occurring in cells other than the phloem-loading CC, and 3)
608 pZmSut1::RFP expression in the sink tissue overlapping with but independent of the

609 sites and mechanisms of phloem unloading. These and other data provide a deeper
610 understanding of the biological functions of *Sut1* in maize, resolve previously conflicting
611 data on the path of Suc unloading in developing grass leaves, provide insights into the
612 evolution of *Sut1* expression and function within grasses, and suggest *Sut1* function was
613 enhanced in the context of the higher Suc export resulting from C₄ photosynthesis.

614

615 In characterizing *ZmSut1* diurnal expression, Aoki et al. (1999) found that *ZmSut1*
616 showed rhythmic expression in juvenile leaf blades, with transcript levels peaking at the
617 end of the d and decreasing during the night. In contrast, we observed no diurnal cycling
618 of *ZmSut1* expression in adult source and sink leaves. There are several possible reasons
619 for this discrepancy. First, Aoki et al. (1999) characterized *ZmSut1* expression in 2-week-
620 old juvenile leaf 3 blade tissues, whereas we investigated expression in 6-week-old adult
621 leaf 11 and immature leaf 17 tissues. Second, Aoki et al. (1999) characterized
622 greenhouse-grown plants while we utilized field-grown materials. Third, we investigated
623 the B73 genotype and Aoki et al. (1999) used a sweet corn variety. Hence, differences in
624 the age of the plants, the growth conditions, and/or the genotypes may contribute to the
625 different results we observed. While likely highly dependent on experimental conditions,
626 our results demonstrate that *ZmSut1* transcript is not regulated diurnally in adult leaves.

627

628 Aoki et al. (1999) also previously determined that feeding Suc through the xylem induced
629 *ZmSut1* expression. Additionally, previous expression analyses and the present one found
630 that *ZmSut1* RNA accumulation mirrors the sink-to-source transition in an emerging leaf.
631 Consistent with these data, pZmSut1::RFPer expression was induced in the CC, PP and
632 XP cells upon leaf maturation in the light and its transition to source tissue. Yet, it was
633 possible that the increased pZmSut1::RFPer expression resulted from leaf age or light-
634 regulated gene expression. However, from experiments analyzing pZmSut1::RFPer
635 expression in variegated *sr2* leaves, we conclusively determined pZmSut1::RFPer
636 expression was not dependent on leaf age or exposure to light but correlates with source
637 tissue. Collectively, the data suggest that pZmSut1::RFPer expression is enhanced upon
638 maturation of the collection phloem and the transition to phloem loading.

639

640 **Function of ZmSUT1 in the collection phloem**

641 Based on the expression of *ZmSut1* in the CC of source leaf veins, we propose it
642 functions within this cell type to load Suc into the collection phloem. This conclusion is
643 based on the results of RNA *in situ* hybridization experiments and on the expression data
644 for both *ZmSut1* transgenes. We also determined ZmSUT1 is localized to the plasma
645 membrane, consistent with its proposed role in apoplasmic phloem loading. We did not
646 detect the expression of *ZmSut1* RNA or the fusion protein in the SE. Although this result
647 is unsurprising given the lack of a nucleus in this cell type, it could have been possible to
648 detect gSUT1-YFP protein that had been transcribed and translated in the CC and then
649 trafficked through PD into the SE, since both wheat TaSUT1 and rice OsSUT1 proteins
650 have been localized to the SE (Aoki et al., 2004; Scofield et al., 2007). Collectively, the
651 localization results suggest the CC and not the SE are the site of Suc uptake into the
652 collection phloem in maize. Once in the CC cytoplasm, Suc would enter into the sieve
653 tube through the PD for long-distance transport. However, it is conceivable the YFP tag
654 on the C-terminus of the ZmSUT1 protein limited protein trafficking from the CC to the
655 SE, as previously suggested for a green fluorescent protein (GFP) fusion of LeSUT1 in
656 tomato (*Solanum lycopersicon*) (Lalonde et al., 2003). However, subsequent
657 immunolocalization experiments indicated LeSUT1 is present in the CC, not the SE
658 (Schmitt et al., 2008). That the transgenic complementation we observed was incomplete
659 may be due to the YFP fusion partially obstructing the ZmSUT1 biochemical function;
660 nevertheless, the YFP fusion must not have compromised the biochemical activity of the
661 protein too severely to provide the level of complementation achieved. Further, it could
662 be argued that the constraint of the ZmSUT1-YFP fusion within the CC instead of the SE
663 could explain that only a partial complementation of the *zmsut1* mutant was observed.
664 Interestingly, fusion of GFP to the C-terminus of AtSUC2, which functions in the CC and
665 therefore does not require trafficking into the SE, expressed under the control of the
666 AtSUC2 promoter was also reduced in its effectiveness of complementing the *atsuc2*
667 mutation (Srivastava et al., 2008). Future experiments to immunolocalize the native
668 ZmSUT1 protein will be necessary to resolve whether it is present in the SE.

669

670 **Function of ZmSUT1 in non-conducting cells within source tissue**

671 An intriguing and surprising finding was that *ZmSut1* is strongly expressed in non-
672 conducting leaf cells (e.g., PP, XP, and BS cells). Indeed, CC expression represented only
673 ~15% of the RNA *in situ* hybridization signal detected within leaf veins. These data
674 suggest this gene likely plays additional roles beyond the canonical one of Suc phloem
675 loading in the leaf blade. A previous study on tobacco (*Nicotiana tabacum*) found the
676 group 2 *NtSUT1* gene was expressed not only in the CC but also in the XP cells of Class I
677 (midrib) through Class IV leaf veins (Schmitt et al., 2008). No expression outside of the
678 CC was found in the Class V veins, the smallest vein class within the leaf. The authors
679 speculated that one potential function for *NtSUT1* expression in the XP cells was to
680 retrieve Suc from the xylem. A similar observation of XP cell expression has been made
681 for group 1 *Suts* in both rice and sugarcane (Rae et al., 2005; Scofield et al., 2007;
682 Ibraheem et al., 2014). As described in the Introduction, from functional and expression
683 studies, rice *OsSUT1* is suggested to function in Suc retrieval into the phloem along the
684 transport path from the leaf blade to the pedicel subtending the grain. Additionally,
685 *OsSut1* expression in XP cells can be induced by aphid feeding, a condition that
686 potentially increases Suc leakage from damaged cells and necessitates Suc retrieval. In
687 sugarcane, *ShSUT1* was expressed in non-phloem cells, where it is proposed to function
688 to prevent Suc loss to the apoplasm (Rae et al., 2005). Hence, these data suggest *OsSUT1*
689 and *ShSUT1* function in non-conducting cells to retrieve Suc from the apoplasm, similar
690 to the proposed role for *ZmSUT1* in cells other than CC.

691

692 Based on the previous and current results, we propose a model for *ZmSut1* function in the
693 leaf blade. This model takes into account the molecular expression, physiological, and
694 genetic data for *ZmSut1* as well as the previous anatomical and radiolabeling work in
695 studies addressing routes of water and Suc movement in the maize plant. Figure 14 shows
696 the schematic of a minor vein in the mature leaf blade. The model proposes that Suc is
697 effluxed from the PP cells by SWEET transporters in the vicinity of the CC/SE complex.
698 *ZmSUT1* functions on the CC plasma membrane to transport Suc into the CC cytoplasm,
699 where it moves through PD into the SE for long-distance transport through the sieve tube
700 to distal sink tissues. Importantly, *ZmSUT1* also functions to retrieve Suc into non-
701 conducting cells (XP, PP, and BS cells) from the vein apoplasm (both phloem and xylem)

702 to recover any Suc not effectively loaded into the CC. ZmSUT1 expression in M cells
703 would similarly function to recover Suc from the leaf apoplasm. Additional evidence in
704 support of the model is enumerated below.

705

706 First, Slewinski et al. (2009, 2010) showed that *zmsut1* mutants had impaired uptake of
707 radiolabeled Suc into the phloem of the leaf blade and that the leaf apoplasm had excess
708 Suc (i.e., Suc droplets), supporting the previous hypothesis of Aoki et al. (1999) that
709 ZmSUT1 functions in the phloem loading of Suc. The present RNA *in situ* hybridization,
710 promoter: reporter gene expression analyses, and full gene translational fusion results
711 showing the expression of *ZmSut1* in the CC provide further support for its role in this
712 process within the collection phloem. To date, maize remains the only grass for which a
713 group 1 *Sut* gene has been shown via expression, biochemical, physiological, and genetic
714 analyses to directly function in Suc phloem loading.

715

716 Second, *ZmSut1* functions in the recovery of Suc from the xylem. When Fritz et al.
717 (1983) exposed maize leaves to $^{14}\text{CO}_2$, the presence of radiolabel was consistently
718 observed in the xylem elements of the small veins, suggesting leakage of photosynthate
719 into the xylem transpiration stream. Additional studies feeding ^{14}C -Suc through the
720 xylem showed that the XP cells were the cells that took up Suc from the xylem (Fritz et
721 al., 1983). Further, a sharp rise in the pH has been observed in the xylem exudate when
722 Suc was fed through the xylem of a detached leaf, indicating the active uptake of Suc
723 from it (Heyser et al., 1978). Both the Suc droplets in the *zmsut1* mutants and the strong
724 expression of *ZmSut1* in the XP cells adjacent to the xylem elements in all of the vein
725 classes suggest that *ZmSut1* is the gene largely responsible for this active uptake of Suc
726 from the xylem.

727

728 Third, ZmSUT1 is proposed to also function in the PP, XP, and BS cells to retrieve Suc
729 not loaded into CC from being eventually lost to the transpiration stream. These other cell
730 types within the vein have abundant PD in their shared cell walls and are therefore
731 symplasmically connected (Evert et al., 1977, 1978). Additionally, the radial and
732 tangential cell walls of the BS cells are suberized, which has been proposed to function to

733 confine Suc within the vein apoplasm (Evert et al., 1977). Hence, ZmSUT1 expression in
734 the PP, XP, and BS cells enables Suc recovery back to the symplasm and thereby
735 provides another opportunity for the plant to efflux the Suc from the PP and load it into
736 the sieve tube for long-distance transport. An intriguing possibility for future study is that
737 the efficiency of Suc transport into the CC vs. Suc uptake from the apoplasm into non-
738 conducting cells could serve as a Suc flux measurement that feeds back to regulate
739 phloem loading.

740

741 One argument against *ZmSut1* functioning in Suc retrieval from the apoplasm in cells
742 other than the CC/SE complexes is that the Suc droplets observed in *zmsut1* mutant
743 leaves solely reflect the failure to perform phloem loading—that is, in wild-type plants,
744 Suc leakage would not occur because ZmSUT1 would load all apoplasmically located
745 Suc into the CC. Hence, any expression of ZmSUT1 in the non-vascular cells would
746 represent a non-functional role. In addition to the strong expression of *ZmSut1* in non-
747 conducting leaf cells, further evidence for ZmSUT1 functioning in Suc retrieval is that
748 we have not observed Suc droplets in other maize mutants with an excessive
749 accumulation of starch and sugars in the leaves, such as *Suc export defective1*,
750 *psychedelic*, and the *tie-dyed1* and *2* mutants (Baker and Braun, 2007; Baker and Braun,
751 2008; Ma et al., 2008; Slewinski and Braun, 2010b). In the *tie-dyed* mutants, the
752 blockage in Suc movement appears to occur between the CC and SE, as evidenced by the
753 CC in the mutants containing a high abundance of oil droplets relative to those in wild-
754 type siblings (Baker et al., 2013). Hence, the process of phloem loading does not appear
755 to be defective, but instead dramatically reduced in the *tie-dyed* mutants. If a
756 consequence of a reduction in phloem loading is the excretion of Suc droplets, we might
757 anticipate observing them in these other mutants.

758

759 If this model of Suc retrieval in the mature leaf blade is accurate, why might the plant
760 maintain such tight control over apoplasmic levels of Suc? In previous studies of rice
761 lines susceptible to pathogenic attack from *Xanthomonas oryzae* pv. *Oryzae*, it was found
762 that bacterial transcription activator-like (TAL) effectors upregulated *OsSWEET11* or
763 *OsSWEET14* in the leaf vascular tissues (Antony et al., 2010; Chen et al., 2010). This

764 upregulation has been proposed to lead to increased Suc release into the apoplasm and
765 thereby facilitate bacterial growth. In resistant lines, these gene promoters are no longer
766 recognized by the TAL effectors (Chu et al., 2006). Thus, one possibility is that the plant
767 maintains apoplasmic Suc concentrations below a certain level as a defense mechanism
768 against pathogenic invasion. Interestingly, the Suc droplets in *zmsut1* mutants also
769 provide evidence that the release of Suc from the PP cells is not feedback regulated
770 (Baker et al., 2012). Hence, if SWEETs are responsible for Suc release into the phloem
771 apoplasm in maize, as proposed for Arabidopsis, ZmSUT1 is presumably a predominant
772 part of the mechanism for controlling Suc apoplasmic levels. Relatedly, ZmSUT1
773 function to load Suc into the collection phloem maintains low apoplasmic Suc levels,
774 which provides a mechanism for homeostatic maintenance of water flow and turgor
775 pressure within the leaf.

776

777 **Functions of ZmSUT1 within sink tissues**

778 pZmSut1::RFP_{er} was expressed in all examined sink tissues throughout the plant,
779 including developing leaves, stems, roots, shoot apical meristems, and developing ears,
780 tassels, and anthers. Expression was invariantly seen in the CC and surrounding non-
781 conducting cells, with high expression levels often observed in the XP cells. With respect
782 to *ZmSut1* function in the transport phloem, we suggest ZmSUT1 retrieves Suc passively
783 leaked from the sieve tube during translocation to maintain the high osmotic potential and
784 hydrostatic pressure gradient in the phloem. This function is analogous to the dual roles
785 proposed for *ZmSut1* function in loading and retrieval in the source tissue. This proposed
786 role has been previously suggested for OsSUT1 and ShSUT1 in the lateral veins of the
787 mature leaf and in the veins of the transport phloem of various tissues (Rae et al., 2005;
788 Scofield et al., 2007). This function would presumably be the primary one for ZmSUT1
789 throughout the transport phloem of the plant. In the stem storage parenchyma cells,
790 ZmSUT1 may function to uptake Suc from the apoplasm during expansive growth, and to
791 retrieve Suc leaked from cells during accumulation and to maintain turgor. A similar
792 function has been proposed during sugar accumulation in sugarcane and sweet sorghum
793 (*Sorghum bicolor*) stems (Bihmidine et al., 2013; Patrick et al., 2013; Bihmidine et al.,
794 2015).

795

796 pZmSut1::RFP_{er} was also expressed in the region of the release phloem, which was
797 marked by symplasmic CF unloading into the developing leaves and roots. In these
798 unloading regions, a portion of the solutes are released from the phloem, but others are
799 transported more distally through it and unloaded apoplastically, as seen in developing
800 leaves. Hence, the transport and release phloems overlap in these regions. Symplasmic
801 phloem unloading has been suggested to be regulated largely by the rate at which Suc
802 moves through the PD into post-phloem sink cells (Patrick, 2012). Suc that is not
803 symplasmically unloaded through PD would continue along the translocation path toward
804 the phloem terminus. We interpret these data to suggest that *ZmSut1*, although expressed
805 in the release phloem, does not directly function to efflux Suc from the phloem as
806 previously postulated. Our findings are consistent with the suggestion that SUT-mediated
807 Suc efflux to the apoplast is unlikely due to thermodynamic considerations (Zhang et
808 al., 2007a). Additionally, these results support the previous data showing symplasmic
809 phloem unloading in maize roots (Giaquinta et al., 1983; Warmbrodt, 1985; Hukin et al.,
810 2002; Ma et al., 2009).

811

812 In the developing ears and tassels, pZmSut1::RFP_{er} expression was most strongly
813 observed in the developing veins and was largely absent in the meristematic tissue, while
814 virtually no expression was observed in the shoot apical meristem. To our knowledge,
815 although Suc movement at the pedicel and nucellus has been assessed at the pre- and
816 post-pollination stages in maize (Porter et al., 1985; Makela et al., 2005; Bihmidine et al.,
817 2013; Tang and Boyer, 2013), the process of Suc unloading in the grass inflorescence at
818 earlier developmental stages remains to be evaluated. Based on the function of group 1
819 *Sut* genes in the transport phloem of various grasses, we speculate that if Suc is unloaded
820 symplasmically into the developing inflorescence tissues, the expression of
821 pZmSut1::RFP_{er} in the veins reflects its function in retrieving Suc leaked into the
822 apoplast. However, if Suc is unloaded into the apoplast (e.g., by SWEET proteins) for
823 subsequent uptake by these sink tissues (either as Suc directly, or as hexoses after
824 cleavage by cell wall invertase), an intriguing idea is that the ZmSUT1 retrieval of Suc
825 back into the phloem competes with the sink cell for Suc (Hafke et al., 2005). Thus, Suc

826 recovery in the phloem might act as a feedback mechanism to signal insufficient sink
827 capacity and excess Suc production to the photosynthetic cells through virtue of a
828 decrease in bulk flow. This mechanism is consistent with the expression reported for
829 grass *Sut1* genes in sink tissues and could be a conserved aspect of its function in these
830 tissues. Future studies will need to be performed to address these various possibilities.

831

832 **Developing maize leaves dynamically switch from symplasmic to apoplasmic phloem** 833 **unloading**

834 Based on anatomical, dye-tracer, and viral movement studies, Haupt et al. (2001)
835 concluded that Suc unloading in the developing barley leaf occurs symplasmically.
836 However, both barley and maize leaves were proposed to use apoplasmic phloem
837 unloading based on ultrastructural studies, which found that the CC/SE complexes in
838 these developing leaves were symplasmically isolated from surrounding cells (Evert and
839 Russin, 1993; Evert et al., 1996b). Our data examining CF unloading into developing
840 maize leaves likely explain the previous discrepancy and reconcile these contradictory
841 reports. We discovered that maize leaves dynamically switch from using symplasmic
842 phloem unloading near the base of the leaf (younger tissue) to apoplasmic phloem
843 unloading in the older regions. Such a dynamic switch in phloem unloading mechanism
844 has been reported previously in many plants and tissues (see Braun et al. (2014) for a
845 review). Hence, we suggest that the barley and maize developing leaves examined by
846 Evert's group for ultrastructural studies were more mature regions employing apoplasmic
847 unloading, while the data of Haupt et al. (2001) reflect symplasmic phloem unloading
848 occurring in younger tissues. This dynamic switch in the phloem unloading mechanism in
849 developing leaves is likely a common feature of grass leaves. More research is necessary
850 to understand the changes during leaf development in PD frequency or conductivity, such
851 as occlusion by callose, that may regulate the symplasmic vs. apoplasmic phloem
852 unloading process.

853

854 **Evolution of group 1 *Suts* in the grasses**

855 Based on the foundational work in rice, expression studies in other grasses, and the
856 present findings, we propose a model for the evolution of group 1 *Sut* genes in the

857 vegetative portions of the plant and suggest the increased importance of *Sut1* function in
858 the evolution of C₄ photosynthesis.

859

860 It is currently hypothesized that the monocot leaf blade evolved from the petiole or the
861 lower leaf zone (leaf base and stipule) of eudicots, with the original leaf lamina present
862 only residually in the tips of the first few seedling leaves (see Slewinski (2013) and
863 references therein). As the petiole flattened, corresponding veins rearranged from a netted
864 to a linear pattern. Within this evolutionary context, the veins formerly contributing to
865 long-distance transport (i.e., transport phloem), now located in the leaf blade, would now
866 function to acquire photosynthate from the photosynthetic cells (i.e., become collection
867 phloem) (Slewinski et al., 2013). A shared trait of group 1 *Sut* genes is their expression in
868 the transport phloem, suggesting that the default role of these genes was to retrieve Suc
869 leaked from the sieve tube along the transport path and potentially in developing tissues.
870 The group 1 *Sut* genes might have replaced the function of other *Sut* genes that may have
871 originally contributed to phloem loading in the former leaf blade (Slewinski et al., 2013).
872 Similarly, the observed expression in the XP cells of transport phloem and sink tissues in
873 the grasses suggests group 1 *Sut* genes could have been co-opted to function for Suc
874 retrieval, particularly in response to breaches in the xylem/phloem barrier. One prediction
875 is that the expression of the group 1 *Sut* genes might have been upregulated to
876 accommodate this increased demand. In this context, it is interesting that we see
877 induction of pZmSut1::RFP expression in the leaf veins upon maturation as source
878 tissue.

879

880 A key consideration in the present study is that Suc leakage from sieve tubes during long-
881 distance transport is a constant challenge to the plant. In previous studies, C₄ grasses
882 (e.g., maize and sorghum) have been shown to have a substantially higher rate of export
883 of photosynthates than C₃ grasses (e.g., wheat and barley) (Grodzinski et al., 1998).
884 Consistent with this idea, Suc content in maize phloem sap is substantially higher than
885 that measured for wheat or rice (Fukumorita and Chino, 1982; Hayashi and Chino, 1986;
886 Ohshima et al., 1990; Weiner et al., 1991). This substantial increase in the translocation
887 of photosynthate would presumably lead to the increased loss of Suc from the phloem in

888 photosynthetic tissues, and would also have demanded a greater reliance on Suc retrieval
889 systems within transport phloem and developing tissues, as the Suc flux increased.
890 Hence, during the shift to C₄ photosynthesis, the group 1 *Sut* genes might have acquired a
891 more imperative role in C₄ plants as compared to C₃ plants. The strong expression of
892 *ZmSut1* and *ShSut1* in non-conducting vascular cells, coupled with the expression of
893 *TaSut1* only in CC/SE, supports this possibility.

894

895 Within the framework of this model, it will be of interest to determine whether variants of
896 sugarcane will express a ShSUT1 homolog in the CC, and whether *shsut1* mutants will
897 also condition a Suc-droplet phenotype. In rice, the absence of an effect on Suc flux in
898 the mature leaf blade of *ossut1* mutants might partly reflect lower photosynthate
899 production in C₃ compared to C₄ grasses. A limitation of this model is that the *Sut1* genes
900 have been studied in only a few grasses. Moreover, only two C₄ grasses are represented
901 in studies of *Sut1* expression and function, and both are of the NADP-malic enzyme type.
902 More studies will need to be performed to address the validity of this hypothesis.

903

904 **Modulating Suc retrieval activity in engineering C₃ grasses for C₄ photosynthesis**

905 The rapidity and frequency of C₄ evolution within the grasses suggest that this group of
906 plants might be pre-adapted for evolving C₄ metabolism (Slewinski, 2013). The
907 expression of group 1 *Sut1* genes within the non-conducting cell types of the leaf blade
908 might represent one such preadaptation for accommodating increased flux from the
909 photosynthetic cells accompanying the higher rates of assimilate export in C₄ plants. Our
910 present results suggest the role of Suc retrieval in the leaf blade is enhanced in maize,
911 potentially as a product of C₄ photosynthesis, resulting in higher levels of Suc production.
912 If so, increased capacity of Suc retrieval may be required for successfully engineering C₃
913 grasses to perform C₄ photosynthesis. If the existing regulatory program for addressing
914 cell damage and Suc leakage in C₃ plants is sufficient for an appropriate response to the
915 increased assimilate flux, the modulation of *Sut1* activity might not be necessary for
916 tailoring C₄ photosynthesis. However, the issue of increased Suc flux will need to be
917 considered if insufficient carbon export occurs or photosynthesis is impaired. If so,

918 further adjustment of *Sut1* regulation might facilitate an improved export rate in C₄-
919 engineered plants.

920

921 **MATERIALS AND METHODS**

922 *Tissue collection, RNA extraction, and cDNA synthesis*

923 Maize (*Zea mays* L.) plants were grown in the field at the University of Missouri South
924 Farm Agricultural Experiment Station. The inbred line B73 was used for the time course
925 experiment. At the v11 stage, approximately 6 weeks after planting, the fully mature leaf
926 11 and the immature leaf 17, which was etiolated and ensconced within the whorl, of 10
927 individual plants were harvested at 4 h intervals over the course of 48 hrs beginning at
928 04:30 am on d 1. Collected tissue was immediately placed in liquid nitrogen and stored at
929 -80°C until processing. 100 mg of frozen leaf tissue was finely ground in a mortar and
930 pestle, total RNA was extracted with Trizol, 50 pg luciferase RNA (Promega, Madison,
931 WI) was added as the reference gene to 1 µg of total RNA, and cDNA was synthesized as
932 described (Bihmidine et al., 2015).

933 *qRT-PCR*

934 *ZmSut1* gene-specific primers were designed and validated according to Bihmidine et al.
935 (2015). Primer sequences and annealing temperatures are listed in Supp. Table S2. For
936 the time course expression analysis, reactions were run in 384-well plates on a CFX384
937 Real Time System (Bio-Rad, Hercules, CA). The d prior to running the qRT-PCR
938 experiment, 4 µL containing 10 ng of cDNA was added to each well of the 384-well plate
939 and then centrifuged. Nuclease-free water was added to a well in place of cDNA as a no
940 template control. The plates were placed in a 30°C incubator overnight to evaporate the
941 water. The following d 5 µL of a reaction mix containing 2.5 µL SsoFast EvaGreen
942 Supermix with low ROX (Bio-Rad, Hercules, CA) and 0.5 µM of both the forward and
943 reverse primers was added. The qRT-PCR experiment was run with the following
944 conditions: 95°C for 30s, with 40 cycles of 95°C for 5s and the appropriate annealing
945 temperature for each primer set for 30s. After 40 cycles, a melt curve analysis was
946 performed to check that a single PCR product was amplified.

947 To quantify *ZmSut1* expression in *zmsut1-m4* mutants compared to wild type, a
948 segregating family was planted in the greenhouse illuminated with supplemental lighting

949 provided by 600-watt high-pressure sodium fixtures under a 16/8 h light: dark regime
950 ($1000 \mu\text{mols m}^{-2} \text{sec}^{-1}$), with the temperatures maintained between 26-31°C during the d
951 and 20-24°C during the night. Individuals were genotyped according to Slewinski et al.
952 (2010). Five individuals homozygous for either the mutant or for the wild-type allele
953 were grown to the v5 stage, upon which the fully expanded fifth leaf was collected and
954 placed into liquid nitrogen. The RNA extraction and cDNA synthesis were performed as
955 outlined above. For the *zmsut1-m4* and wild-type expression analysis, the 10 μL reaction
956 mix consisted of 10 ng cDNA, 5 μL SsoFast EvaGreen Supermix with low ROX (Bio-
957 Rad, Hercules, CA), and 0.5 μM of both *ZmSut1* or luciferase forward and reverse
958 primers (Supp. Table S2).

959 Quantitative cycle values were determined using a regression method and were analyzed
960 using the standard curve method (Larionov et al., 2005). The time course experiment
961 consisted of 10 individual (biological) samples for each time point with 5 technical
962 replicates each, whereas the *zmsut1-m4* expression experiment was composed of 5
963 biological replicates for each genotype with 4 technical replicates each. The standard
964 curve used for the time course analysis was composed of a pool of cDNA from each
965 individual at each time point, while the standard curve for *zmsut1-m4* expression was
966 composed of a cDNA pool from the wild-type individuals in the experiment. Statistically
967 significant differences at $p < 0.05$ were determined using Proc GLM (SAS v9.3).

968 *RNA In Situ Hybridization*

969 Small tissue segments ($3 \times 1 \text{ mm}$) dissected from fully emerged leaves 12 and 13 of
970 greenhouse-grown 13-week-old B73 and *zmsut1-m4* mutant plants were fixed overnight
971 in ice-cold acetone, dehydrated through an acetone/xylene series, and embedded in
972 paraffin, as described (Zhang et al., 2007b). The embedded tissue was sectioned at a
973 thickness of 12 μm and adhered to glass slides on heating plates. The subsequent probe
974 selection, hybridization, and fast-blue color development were conducted by Affymetrix
975 (San Diego, CA, USA) as described (Bowling et al., 2014). ImageJ was used to compare
976 the relative percentage of signal within the CC/SE to the signal in the PP, XP, and BS
977 cells in lateral ($n = 5$), intermediate ($n = 5$), and small ($n = 10$) veins using the “Color
978 Pixel Counter” plug-in (<http://rsb.info.nih.gov/ij/>). The xylem vessel elements in all vein

979 classes, the epidermis, and the HS cells in the lateral and intermediate veins were
980 excluded from the analysis.

981 *Transgenic plants*

982 Maize lines carrying transgenic constructs expressing 1) RFP targeted to the endoplasmic
983 reticulum under the control of the *ZmSut1* promoter region (pZmSut1::RFP_{ER}), 2) YFP
984 translationally fused to the C-terminus of the ZmSUT1 protein using the full-length
985 *ZmSut1* genomic sequence (gSUT1-YFP), and 3) CFP translationally fused to the N-
986 terminus of PIP2-1 (AQUAPORIN) using the *PIP2-1* genomic sequence (PIP2-1-CFP)
987 were obtained from the Maize Cell Genomics Project, and were constructed as described
988 at <http://maize.jcvi.org/cellgenomics/index.php>. These constructs were backcrossed into
989 the B73 inbred line at least 3 times prior to analyses. Plants carrying YFP and RFP
990 transgenes were PCR-genotyped using primers listed in Supp. Table S2, or plants
991 carrying the transgenes (YFP, RFP, or CFP) were visually identified by fluorescent
992 microscopy.

993 For the etiolated seedling light-shift experiment, a family segregating for
994 pZmSut1::RFP_{ER} was germinated in the dark for 8 d, at which time leaf 1 and the tip of
995 leaf 2 had emerged from the coleoptile. The etiolated seedlings were brought into the lab,
996 and a small portion of leaf 1 was harvested from one side of the midrib for microscopy.
997 The plants were left under dim fluorescent white lighting (5 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) for 48 hrs to
998 induce chlorophyll synthesis, then moved into the greenhouse to transition to source
999 tissues. After 5 d, tissue was harvested from leaf 1 opposite to the location of the initial
1000 sampling.

1001 For the *sr2* leaf variegation experiment, plants carrying the pZmSut1::RFP_{ER} construct
1002 were used as males and backcrossed twice to *sr2* mutants.

1003 For the transgenic complementation test of gSUT1-YFP, plants carrying the transgene
1004 were crossed to plants heterozygous for the *zmsut1-m1* mutant allele (Slewinski et al.,
1005 2009). Plants carrying the transgene and *zmsut1-m1* allele were identified by genotyping
1006 (Rotsch et al., 2015; Leach et al., 2016) and backcrossed to *ZmSut1/zmsut1-m1*
1007 heterozygous plants to generate families for analyses. Morphometric and statistical
1008 analyses were conducted as described (Braun et al., 2006; Baker and Braun, 2008; Ma et
1009 al., 2008).

1010 *Light, Fluorescent, and Confocal Microscopy of Reporter Lines*

1011 For each type of illumination within a figure, all images were captured using identical
1012 microscope and camera settings, unless otherwise noted. Bright-field and epi-
1013 fluorescence microscopy of organs and tissues from plants expressing the
1014 pZmSut1::RFP_{er}, gSUT1-YFP, and PIP2-1-CFP constructs were performed on a Nikon
1015 Eclipse 80i microscope equipped with a 100-W mercury bulb and a DXM1200F camera
1016 (Huang et al., 2009). Filter cubes used were: UV (360- to 370-nm excitation filter and a
1017 420-nm long-pass emission filter), CFP (412- to 462-nm excitation filter and a 460- to
1018 500-nm band-pass emission filter), YFP (465- to 495-nm excitation filter and a 515- to
1019 555-nm band-pass emission filter), and RFP (530- to 560-nm excitation filter and a 590-
1020 to 650-nm band-pass emission filter). The excitation peaks for chlorophyll *a* (430, 662
1021 nm in methanol) and *b* (453, 642 nm in methanol) occurred well outside the range of the
1022 RFP filter (530-560 nm), resulting in the virtual absence of chlorophyll autofluorescence
1023 and allowing a simple assessment of RFP signal within leaves. For examination, shoot
1024 apical meristems were dissected from 2-week-old greenhouse-grown seedlings; roots,
1025 developing leaves, and stems from 6-week-old greenhouse-grown plants; developing
1026 tassels from 8-week-old greenhouse-grown plants; and maturing tassels and developing
1027 ears from 10-week-old greenhouse-grown plants. For examining mature leaf tissue,
1028 transverse hand-cut or paradermal sections were generated using a razor blade and
1029 mounted in dH₂O, while reproductive structures and meristems were whole-mounted in
1030 dH₂O after dissection from the plant. Images were captured using Nikon NIS Elements F
1031 software (version 3.0).

1032 For visualizing RFP expression in an emerging leaf 5 of 2-week-old seedlings carrying
1033 the pZmSut1::RFP_{er} construct, we used a Leica MZFLIII dissecting stereomicroscope
1034 equipped with a dsRed-bandpass filter (Leica Microsystems, Bannockburn, IL) and a 12-
1035 bit color CCD camera (Optronics Laboratories, Inc., Goleta, CA). The plants were
1036 examined when the tip of leaf 5 was just emerging from the whorl. The leaf was dissected
1037 from the plant and divided into ten 10-mm segments, and representative regions from
1038 each leaf segment were photographed. All photographs were taken using the same
1039 exposure time, microscope, and camera settings.

1040 A Zeiss 510 META laser scanning confocal microscope (Carl Zeiss Microscopy, LLC)
1041 was used to evaluate the cellular expression of the pZmSut1::RFPper construct in mature
1042 leaves and internodes. RFP was excited with a 543-nm HeNe laser, and fluorescence was
1043 recorded using a 565- to 615-nm band-pass filter. Chloroplast autofluorescence was
1044 induced by a 488-nm argon laser line and recorded using a 650- to 710-nm band-pass
1045 filter. For visualization of the cell walls, the samples were stained with 0.005% aniline
1046 blue (w/v) in 0.15 M potassium phosphate buffer (pH 8.2) (Ruzin, 1999) and excited with
1047 a 458-nm argon laser line, with fluorescence recorded using a 535- to 590-nm band-pass
1048 filter.

1049 For evaluating gSUT1-YFP subcellular localization alone or in double-labelled lines
1050 relative to PIP2-1-CFP or pZmSut1::RFPper, a Zeiss TCP SP8 MP inverted spectral
1051 confocal microscope with a tunable white laser and fixed visible laser lines was used with
1052 the following settings: YFP (tunable white laser light): excitation, 514 nm; emission band
1053 path, 525 to 575 nm; RFP (tunable white laser light): excitation, 584 nm; emission band
1054 path, 590 to 660 nm; UV (405-nm laser line): excitation, 405 nm; emission band path,
1055 420 to 500 nm; CFP (458-nm argon-laser line): excitation, 458 nm; emission band path,
1056 465 to 520 nm; chlorophyll autofluorescence (tunable white laser light): excitation, 488
1057 nm; emission band path, 650 to 800 nm. UV excitation was used to visualize the cell
1058 walls.

1059 To further investigate the subcellular localization of the gSUT1-YFP translational fusion
1060 protein, we performed live-cell imaging of paradermal leaf sections with an Olympus IX-
1061 71 inverted microscope (Center Valley, PA) equipped with a Yokogawa CSU-X1 5000-
1062 rpm spinning disc unit (Tokyo, Japan), Andor iXon Ultra 897 High Speed EMCCD
1063 camera (Belfast, United Kingdom), PZ-2000 XYZ series automated stage with Piezo Z-
1064 axis top plate (Applied Scientific Instrumentation, Eugene, OR), and a 60 \times -silicon oil
1065 objective (Olympus UPlanSApo 60 \times /1.30 Sil), as described in Smith et al. (2014). YFP
1066 was excited with a Spectra Physics 515-nm diode laser (Santa Clara, CA), with
1067 fluorescence collected through a 488-, 515-, and 561-nm dichroic beamsplitter
1068 (ZT405/514/561TPC-XR; Chroma Technology Corp., Rockingham, VA) and 515–569-
1069 nm band-pass filter (FF01-542/27-25; Semrock Brightline, Rochester, NY). For verifying
1070 that gSUT1-YFP localized to the plasma membrane, paradermal leaf sections were

1071 plasmalysed by exposure to a solution of 0.75 M NaCl for 10 minutes and then mounted
1072 for examination. Images were captured using Andor iQ2 software (Belfast, United
1073 Kingdom).

1074 *CFDA Dye Movement Assays*

1075 A solution of CFDA in water (50 µg/ml) was prepared from a stock solution as
1076 previously described (Bihmidine et al., 2015). For the root studies, the cut end of the fully
1077 mature leaf 4 of 10-week-old greenhouse-grown plants expressing the pZmSut1::RFPer
1078 construct was submerged in a 30-mL solution of CFDA in a 50-mL conical tube for 1 h.
1079 Afterwards, the dye was allowed to move through the plant for an additional 3 hrs. The
1080 tips of aerial prop roots that penetrated the soil were harvested, cut longitudinally through
1081 the center under a dissecting scope with a razor blade, and assessed for CF signal with
1082 fluorescence microscopy, as previously described (Bihmidine et al., 2015). For
1083 examining phloem unloading in developing sink leaves, a mature source leaf 9 of a 38-d-
1084 old pZmSut1::RFPer transgenic plant was fed CF for 20 min, followed by translocation
1085 within the plant for 5 hrs prior to tissue harvest and analysis. Cross-sections from the
1086 enclosed developing sink leaves were taken at 50% and 25% of the distance between the
1087 base and top of the sheath of the fed leaf.

1088

1089 **Supplemental Material**

1090 Figure S1. *zmsut1* mutant leaves excrete droplets of Suc.

1091 Figure S2. qRT-PCR analysis of *zmsut1-m4* homozygous mutant and wild-type leaves.

1092 Figure S3. Expression of pZmSut1::RFPer in a leaf undergoing the sink-to-source
1093 transition.

1094 Figure S4. gSUT1-YFP exhibits the same cellular expression pattern as pZmSut1::RFPer.

1095 Table S1. Morphometric analyses of gSUT1-YFP transgenic complementation test.

1096 Table S2. List of PCR primers used.

1097

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1106 of the spinning disc confocal equipment. We thank Wilson Lew, Erik Hasal, and
1107 Maureen Hughes from Affymetrix for technical assistance with the *in situ*.
1108

1109 **Tables**

1110 Table 1. Quantification of *ZmSut1* RNA *in situ* hybridization signal in different cell types
1111 within vein classes

1112

Vein Class	Blue pixels in CC/ total blue pixels	Blue pixels in VP/ total blue pixels	Blue pixels in BS/ total blue pixels	Ratio VP+BS /CC
Lateral	13.6±3.0	81.1±3.6	5.4±2.7	8.3±2.2
Intermediate	16.3±6.4	55.2±9.8	27.1±5.5	6.2±3.8
Small	12.6±9.3	60.0±18.3	27.4±15.9	11.0±8.1

1113

1114 Blue pixels within each vein were identified in companion cells (CC), vascular
1115 parenchyma (VP) cells, or bundle sheath (BS) cells. Numbers of blue pixels are the
1116 average percent values for the specified cell type within each vein class ± the standard
1117 deviation. The last column is the ratio of blue pixels in the VP and BS cells compared to
1118 the CC.

1119

1120 **Figure legends**

1121 **Figure 1.** *ZmSut1* expression is stable and does not cycle diurnally in adult maize leaves.
1122 qRT-PCR expression of *ZmSut1* in B73 mature and immature leaves over 48 hrs.
1123 Samples were harvested every 4 hrs. Measurements are average expression values for 10
1124 biological samples of *ZmSut1* relative to exogenously supplied luciferase mRNA used as
1125 a normalization control. Values are relative units. Red squares indicate mature leaf 11
1126 source tissue and blue diamonds indicate immature leaf 17 sink tissue. Error bars show
1127 standard error.

1128

1129 **Figure 2.** RNA *in situ* hybridization demonstrates that *ZmSut1* is expressed in the CC,
1130 XP, PP, and BS cells of mature leaf blades. Expression is revealed by the blue precipitate.
1131 A, B. Transverse section through a B73 leaf showing the anatomy of a lateral vein (left)
1132 and a small vein (right) under bright-field (A) and UV autofluorescence (B). The
1133 different cell types are labelled: BS, bundle sheath, CC, companion cell, E, epidermis,
1134 HS, hypodermal sclerenchyma, M, mesophyll, MX, metaxylem element, PP, phloem
1135 parenchyma, PX, protoxylem lacunae, SE, sieve element, XP, xylem parenchyma. C, D,
1136 F. Wild-type (WT) B73 mature leaf sections hybridized with *ZmSut1* probe. G. *zmsut1*
1137 mutant leaf section hybridized with *ZmSut1* probe. E, H. Wild-type B73 mature leaf
1138 sections developed without probe. C, E show small veins, D shows an intermediate vein,
1139 and F-H show lateral veins. Black arrows point to CC; red arrows to SE; blue arrows to
1140 BS cells; arrowheads to large XP cells. Scale bar = 50 μ m.

1141

1142 **Figure 3.** The pZmSut1::RFPper transcriptional reporter gene recapitulates *ZmSut1*
1143 expression observed by RNA *in situ* hybridization. A-I. Confocal images showing
1144 expression of pZmSut1::RFPper transgene in transverse and paradermal leaf sections. A.
1145 Transverse section of a minor vein showing RFP is expressed in the CC (white arrow),
1146 XP (arrowhead), PP, and to a more limited extent in the BS cells (yellow arrow). The cell
1147 outlines were visualized with aniline-blue staining; the green signal represents
1148 chlorophyll autofluorescence. B. Transverse section of a minor vein from a plant lacking
1149 the transgene. C. Transverse section of a lateral vein showing RFP expression in CC, XP,
1150 PP, and to a lesser extent in BS cells. Blue signal shows cell walls and green signal is

1151 chlorophyll autofluorescence. D. Same image as in C, but only showing RFP expression.
1152 E. Transverse section of a lateral vein of a non-transgenic control showing no RFP
1153 expression. Blue signal shows cellular anatomy. F. Paradermal section of a minor vein
1154 showing RFP expression in the CC, PP, and to a lesser extent in the BS cells. G.
1155 Paradermal section of non-transgenic control showing no RFP expression. Blue signal
1156 shows cell walls and green signal is chlorophyll autofluorescence. H. Same section as in
1157 F showing only RFP signal. I. Same section as in G showing only red channel. BS,
1158 bundle sheath cell, CC, companion cell, PP, phloem parenchyma cell. Scale bar = 25 μm .
1159

1160 **Figure 4.** pZmSut1::RFP_{er} is expressed early in vein development in sink leaves. Epi-
1161 fluorescence microscope images of pZmSut1::RFP_{er} expression in developing leaves. A-
1162 J. Transverse cross-sections through inner developing leaves of 2-week-old seedlings. A,
1163 B. Non-transgenic control sections. Panel A shows autofluorescence of HS cell walls. C-
1164 J. Transverse sections of pZmSut1::RFP_{er} transgenic leaves located approximately half-
1165 way between the blade-sheath boundary and the base of the enclosing mature leaf (C, D),
1166 at the base of immature blade tissue (E, F), in immature sheath tissue (G, H), and located
1167 just above the meristem (I, J). Panels C, E, G, I, K, M show RFP images. Panels B, D, F,
1168 H, J, L, N show UV autofluorescence images. K, L. Close up of the lateral vein in middle
1169 of panels C, D. M, N. Close up of a developing lateral vein in panels E, F. Expression is
1170 observed in the protophloem (white arrow). Note that with the RFP filter cube, at these
1171 exposure settings, virtually no red signal from chlorophyll is detected (cf. Fig. 4A, B).
1172 Scale bar in A-J = 100 μm . Scale bar in K-N = 25 μm .

1173
1174 **Figure 5.** pZmSut1::RFP_{er} expression in leaves is induced in veins upon shifting plants
1175 from growth in the dark to the light. A, B. RFP images. C, D. UV autofluorescence
1176 images. A, C show an etiolated sink-leaf cross-section. B, D show a leaf cross-section
1177 after shifting plants into the light and the leaf matured as source tissue. Note that with the
1178 RFP filter cube, at these exposure settings, virtually none of the red signal is from
1179 chlorophyll. Scale bar = 100 μm .

1180

1181 **Figure 6.** pZmSut1::RFP expression is induced in veins of mature source tissue
1182 compared with albino sink tissue in variegated *sr2* mutant leaves. A, C, E show a cross-
1183 section through a green-white border of a *sr2* leaf expressing pZmSut1::RFP. B, D, F
1184 show a non-transgenic control variegated *sr2* mutant leaf. A, B. Bright-field. C, D. RFP
1185 signal. E, F. UV autofluorescence. Note that with the RFP filter cube, at these exposure
1186 settings, virtually no red signal from chlorophyll is detected (cf. Fig. 6D, F). Scale bar =
1187 100 μ m.

1188

1189 **Figure 7.** pZmSut1::RFP expression is broad initially in developing stem, but becomes
1190 restricted in mature stem veins. Transverse sections showing expression of the
1191 pZmSut1::RFP transgene in immature (A) and mature stem (C). B. UV
1192 autofluorescence of tissue shown in panel A. D. Transverse section of mature stem of
1193 non-transgenic control. A, C, D. RFP channel. A. Expression of the transgene is initially
1194 strongest in the protoxylem and protophloem, with lower signal in the developing
1195 parenchyma cells. C. At maturity pZmSut1::RFP expression is highest in veins, with
1196 low level in the storage parenchyma. E-G. Confocal images of mature stem vein showing
1197 pZmSut1::RFP expression in the XP cells (arrowhead) and CC (arrow). H-J. Confocal
1198 images of mature stem vein of non-transgenic control exhibiting autofluorescence. E, H.
1199 RFP channel. F, I. UV autofluorescence showing cell walls. G. Merged image of E and F.
1200 J. Merged image of H and I. Note that with the RFP filter cube, at these exposure
1201 settings, virtually no red signal is detected from chlorophyll (cf. Fig. 7C, D). Scale bar in
1202 A, B = 250 μ m; in C, D = 500 μ m; in E-J = 25 μ m.

1203

1204 **Figure 8.** pZmSut1::RFP displays broad expression in multiple vegetative and
1205 reproductive sink tissues. Epi-fluorescent microscope images of pZmSut1::RFP
1206 expression in the shoot apical meristem, in developing tassels and ears, and in developing
1207 roots. A, C, E, G, I, K, M, N, O, Q, S, U. RFP signal. B, D, F, H, J, L, P, R, T, V. UV
1208 autofluorescence of corresponding tissue. A, B. Shoot apical meristem. C, D. Developing
1209 tassel. E-L. Developing ear. G-L represent cross-sections through the developing ear. M,
1210 N. Maturing tassel. O-V. Developing root. O. Transverse section near the root tip
1211 showing RFP expression is largely restricted to the phloem (arrow) and xylem

1212 (arrowhead). Q. Section slightly higher than that of O showing RFP expression in the
1213 phloem and developing xylem elements. S. Section at cusp between developing and
1214 mature xylem cells. Arrowhead indicates xylem element presumably undergoing
1215 autolysis. Arrow indicates phloem. U. Mature root. RFP expression can be seen in the
1216 phloem and diffusely throughout the root. Scale bar = 100 μm for A, B, K, L, O-V; 250
1217 μm for C-J, N; 500 μm for M. Exposure times for panels A, E, G, I, N = 750 ms; for C =
1218 4 s; for K, M = 2 s. for O, Q, S, U = 1 s.

1219

1220 **Figure 9.** pZmSut1::RFP expression pattern differs from that of the phloem unloading
1221 zone identified by CF efflux into cortical cells of a pZmSut1::RFP transgenic root. A,
1222 E. UV autofluorescence. B, F. CF signal. White arrow indicates region of CF efflux from
1223 phloem into cortical cells. C, G. RFP signal. D, H. Overlay of the CF and RFP signals. E,
1224 F, G, and H are closer views of A, B, C, and D, respectively. Scale bar in A-D = 500 μm ;
1225 in E-H = 250 μm .

1226

1227 **Figure 10.** Developing leaves exhibit either symplasmic or apoplasmic phloem unloading
1228 in distinct regions that overlap pZmSut1::RFP expression. A, C, E. Cross-section taken
1229 approximately half-way up the blade of a developing pZmSut1::RFP expressing sink
1230 leaf. B, D, F. Cross-section taken approximately a quarter-way up the blade from the base
1231 of the same developing sink leaf. A, B. UV autofluorescence. C, D. CF signal. C. Arrow
1232 shows CF confinement within the symplasmically isolated phloem. D. Arrowhead shows
1233 vein symplasmically unloading CF into adjacent cells. CF movement marked by white
1234 bracket. E, F. RFP signal. Scale bar = 100 μm .

1235

1236 **Figure 11.** The gSut1-YFP transgene largely complements the *zmsut1* mutant phenotype.
1237 *zmsut1* homozygous mutant plants carrying the transgene (middle) grew to near wild-type
1238 height (left), and produced tassels that shed pollen and ears that produced silks. By
1239 contrast, the *zmsut1* homozygous mutants (right) that lacked the transgene and survived
1240 were stunted and typically failed to undergo anthesis or produce ears.

1241

1242 **Figure 12.** A ZmSUT1 protein translational fusion shows the same cellular expression
1243 pattern as observed with RNA *in situ* hybridization. Confocal images of the ZmSUT1
1244 protein translationally fused at the C-terminus with YFP in transverse and paradermal
1245 leaf sections. A, B. Transverse section of a leaf lateral vein showing gSUT1-YFP is
1246 expressed in CC (arrows), XP (arrowheads), PP, and BS cells (blue arrow). Asterisks
1247 indicate SE. C. Paradermal section of a leaf minor vein showing gSUT1-YFP expression.
1248 A, C. YFP signal. B. Combined YFP, cell wall autofluorescence in blue, and chlorophyll
1249 autofluorescence in red. BS, bundle sheath cells, CC, companion cells, PP, phloem
1250 parenchyma cells, SE, sieve element. Scale bar = 25 μ m.

1251

1252 **Figure 13.** ZmSUT1 localizes to the plasma membrane. Confocal images of the
1253 expression of the SUT1 protein fused at the C-terminus with YFP. A-C. Paradermal
1254 section of a leaf minor vein focused on the BS cells. A. YFP signal. Arrowheads indicate
1255 YFP localization in two adjacent cells, separated by their shared cell wall. B. Combined
1256 YFP and chloroplasts (green). C. Combined YFP, chloroplasts, and cell wall (blue). D-F,
1257 Paradermal section of a leaf minor vein focused on the BS cells of a gSUT1-YFP and
1258 PIP2-1-CFP transgenic plant. D. YFP signal. E. CFP signal. F. Combined YFP and CFP
1259 signal. G-I. Spinning disc confocal image supporting ZmSUT1-YFP plasma membrane
1260 localization. G. Pre-plasmolysis YFP signal located at cell periphery. H. After plasmolysis
1261 with 0.75 M NaCl the plasma membrane has retracted; however, the plasma membrane is
1262 attached to the cell wall at the PD, resulting in the Hechtian strands (arrows). I. Non-
1263 transgenic control section. Scale bar = 10 μ m for A-C; = 25 μ m for D-I.

1264

1265 **Figure 14.** Model for dual functions of *ZmSut1* in phloem loading and retrieval. Red
1266 arrow indicates ZmSUT1 loading Suc into CC. Purple arrows indicate ZmSUT1
1267 retrieving Suc into non-conducting vascular cells. Yellow arrows show symplasmic Suc
1268 movement. Grey rectangles represent symplasmic connectivity through PD. Light blue
1269 rectangles with black arrows represent SWEET proteins effluxing Suc to the apoplast of
1270 PP cells. Beige color represents vein apoplast. BS, bundle sheath cell, CC, companion
1271 cell, M, mesophyll cell, PP, phloem parenchyma cell, SE, sieve element, TST, thick-
1272 walled sieve element, XE, xylem element, XP, xylem parenchyma cell.

1273 **Supplementary Material**

1274 **Supplemental Figure S1.** The hydathodes of *zmsut1* mutant leaves excrete droplets with
1275 high concentrations of Suc (left).

1276 **Supplementary Figure S2.** qRT-PCR analysis shows that the *zmsut1-m4* homozygous
1277 mutant leaves (mut) express a very low level of *ZmSut1* transcript compared to wild-type
1278 (WT) siblings.

1279 **Supplemental Figure S3.** Expression of the pZmSut1::RFPper transgene in an emerging
1280 leaf (leaf 5) undergoing the sink-to-source transition.

1281 **Supplemental Figure S4.** gSUT1-YFP exhibits the same cellular expression pattern as
1282 pZmSut1::RFPper.

1283

1284 **Supplemental Figure S1.** The hydathodes of *zmsut1* mutant leaves excrete droplets with
1285 high concentrations of Suc (left). The droplets dry to form Suc beads (arrowhead).
1286 Equivalent excretions are not observed in the wild-type leaf (right).

1287

1288 **Supplementary Figure S2.** qRT-PCR analysis shows that the *zmsut1-m4* homozygous
1289 mutant leaves (mut) express a very low level of *ZmSut1* transcript compared to wild-type
1290 (WT) siblings. Values are relative units. Error bars are standard error. Asterisk indicates
1291 statistically significant difference at $p < 0.05$.

1292

1293 **Supplemental Figure S3.** Expression of the pZmSut1::RFPper transgene in an emerging
1294 leaf (leaf 5) undergoing the sink-to-source transition. The tip of the leaf (A) has emerged
1295 out of the whorl into the light. Each panel A-J is an image taken from a consecutive 1 cm
1296 segment of the leaf. The bottom segment at the leaf base (J) represents the region 10 cm
1297 proximal from the leaf tip. The grey triangle represents the gradient of exported carbon
1298 from the source tissue. The physiological, anatomical, and developmental events
1299 indicated are approximately positioned, correspond coarsely to cross-sections shown in
1300 Fig. 4, and are extrapolated from Evert et al. (1996a). Scale bar = 1 mm for all panels.

1301

1302 **Supplemental Figure S4.** gSUT1-YFP exhibits the same cellular expression pattern as
1303 pZmSut1::RFPper. A-D. Paradermal section of a leaf minor vein of a gSUT1-YFP;

1304 pSut1::RFP_{er} transgenic plant. E-H. Paradermal section of a leaf minor vein of a non-
1305 transgenic control sibling plant. A, E. YFP signal. B, F. RFP signal. C, G. Combined YFP
1306 and RFP signals. D, H. Combined YFP, RFP, chlorophyll, and UV autofluorescence
1307 signals. Scale bar = 25 μ m.
1308

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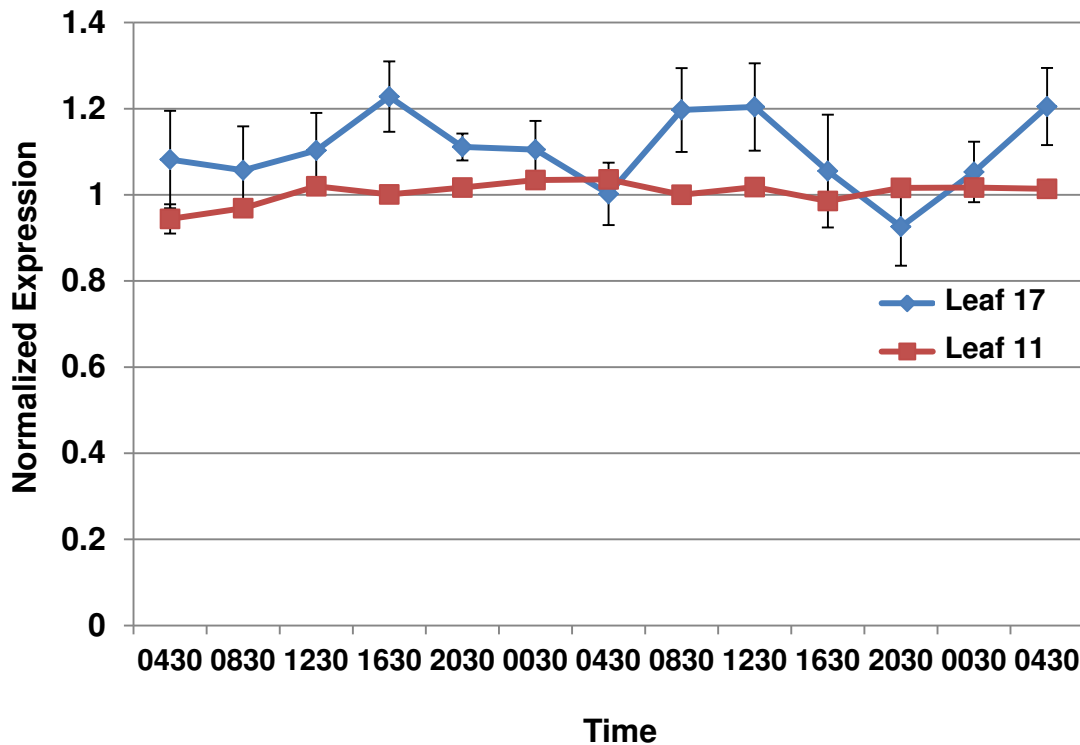


Figure 1. ZmSut1 expression is stable and does not cycle diurnally in adult maize leaves. qRT-PCR expression of ZmSut1 in B73 mature and immature leaves over 48 hrs. Samples were harvested every 4 hrs. Measurements are average expression values for 10 biological samples of ZmSut1 relative to exogenously supplied luciferase mRNA used as a normalization control. Values are relative units. Red squares indicate mature leaf 11 source tissue and blue diamonds indicate immature leaf 17 sink tissue. Error bars show standard error.

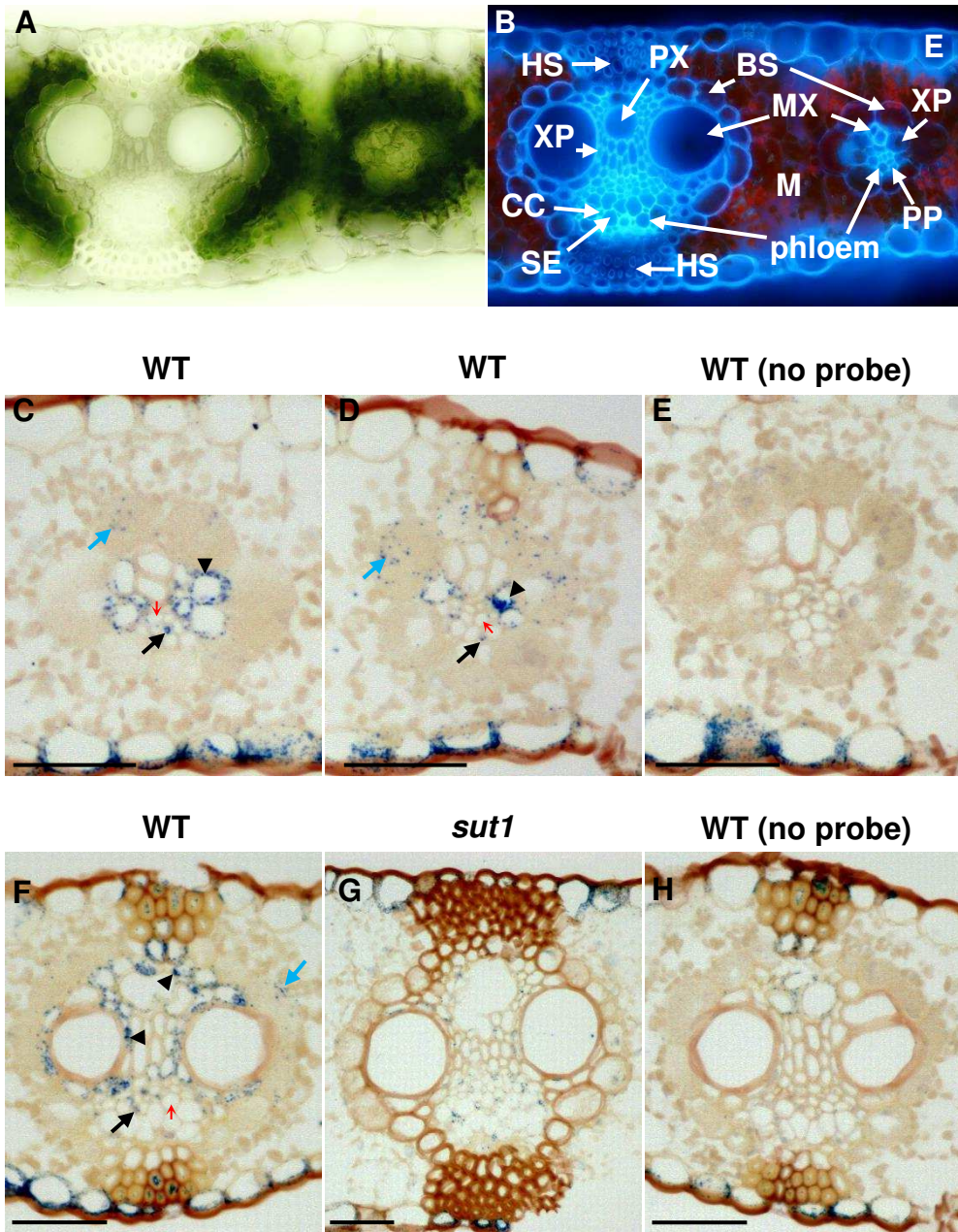


Figure 2. RNA in situ hybridization demonstrates that *ZmSut1* is expressed in the CC, XP, PP, and BS cells of mature leaf blades. Expression is revealed by the blue precipitate. A, B. Transverse section through a B73 leaf showing the anatomy of a lateral vein (left) and a small vein (right) under bright-field (A) and UV autofluorescence (B). The different cell types are labelled: BS, bundle sheath, CC, companion cell, E, epidermis, HS, hypodermal sclerenchyma, M, mesophyll, MX, metaxylem element, PP, phloem parenchyma, PX, protoxylem lacunae, SE, sieve element, XP, xylem parenchyma. C, D, F. Wild-type (WT) B73 mature leaf sections hybridized with *ZmSut1* probe. G. *zmsut1* mutant leaf section hybridized with *ZmSut1* probe. E, H. Wild-type B73 mature leaf sections developed without probe. C, E show small veins, D shows an intermediate vein, and F-H show lateral veins. Black arrows point to CC; red arrows to SE; blue arrows to BS cells; arrowheads to large XP cells. Scale bar = 50 μ m.

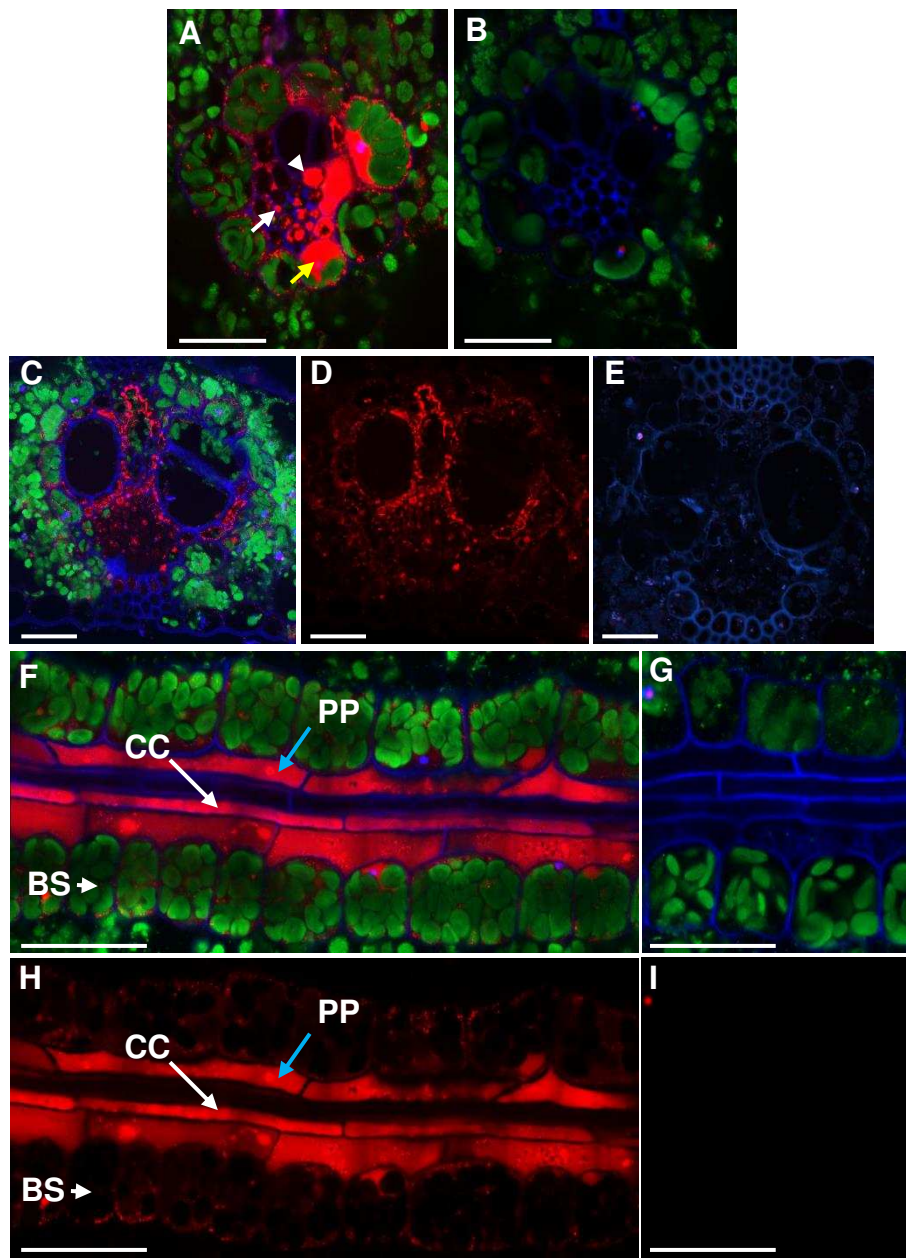


Figure 3. The pZmSut1::RFP transcriptional reporter gene recapitulates ZmSut1 expression observed by RNA in situ hybridization. A-I. Confocal images showing expression of pZmSut1::RFP transgene in transverse and paradermal leaf sections. A. Transverse section of a minor vein showing RFP is expressed in the CC (white arrow), XP (arrowhead), PP, and to a more limited extent in the BS cells (yellow arrow). The cell outlines were visualized with aniline-blue staining; the green signal represents chlorophyll autofluorescence. B. Transverse section of a minor vein from a plant lacking the transgene. C. Transverse section of a lateral vein showing RFP expression in CC, XP, PP, and to a lesser extent in BS cells. Blue signal shows cell walls and green signal is chlorophyll autofluorescence. D. Same image as in C, but only showing RFP expression. E. Transverse section of a lateral vein of a non-transgenic control showing no RFP expression. Blue signal shows cellular anatomy. F. Paradermal section of a minor vein showing RFP expression in the CC, PP, and to a lesser extent in the BS cells. G. Paradermal section of non-transgenic control showing no RFP expression. Blue signal shows cell walls and green signal is chlorophyll autofluorescence. H. Same section as in F showing only RFP signal. I. Same section as in G showing only red channel. BS, bundle sheath cell, CC, companion cell, PP, phloem parenchyma cell. Scale bar = 25 μm .

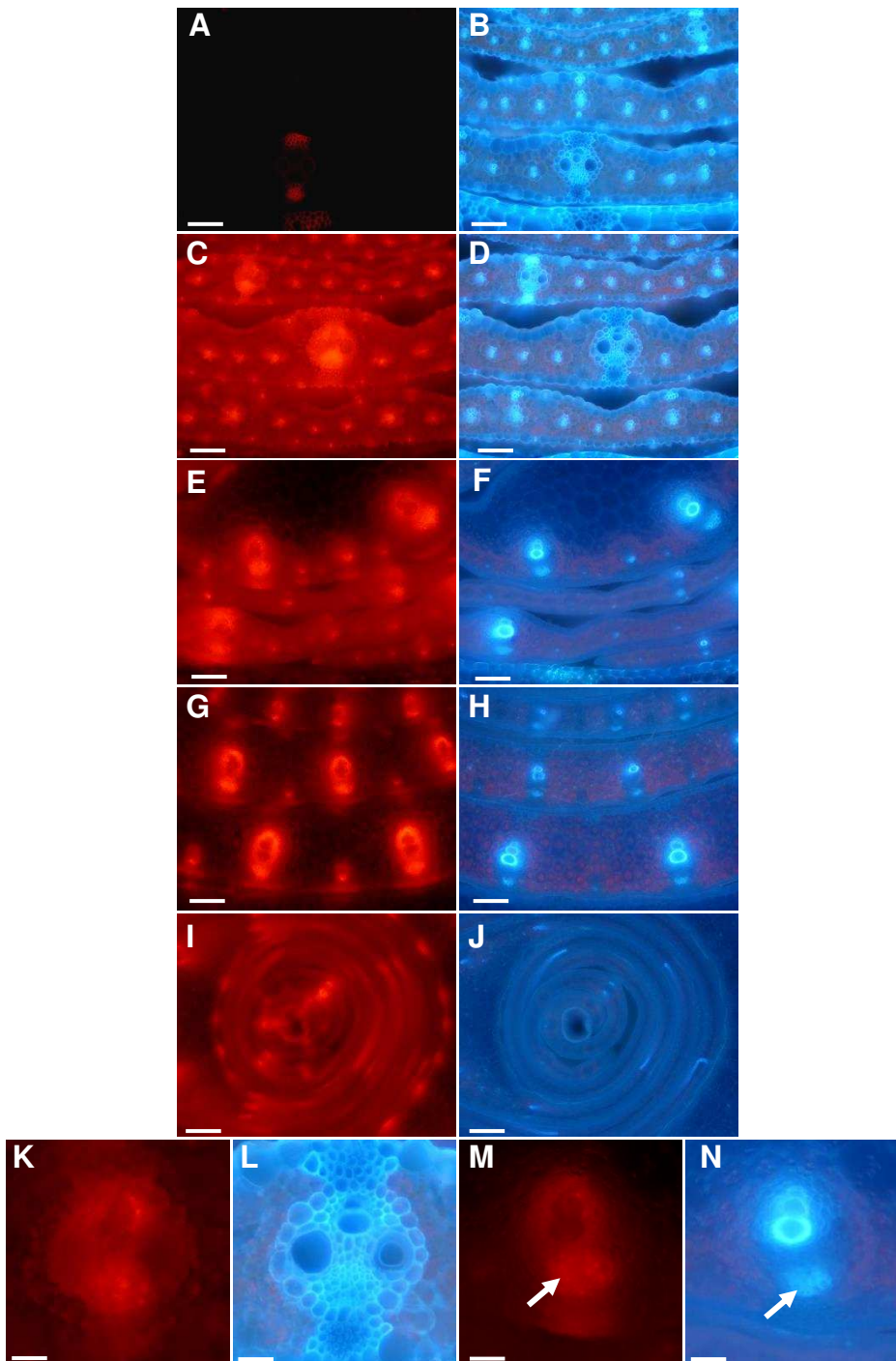


Figure 4. pZmSut1::RFP is expressed early in vein development in sink leaves. Epi-fluorescence microscope images of pZmSut1::RFP expression in developing leaves. A-J. Transverse cross-sections through inner developing leaves of 2-week-old seedlings. A, B. Non-transgenic control sections. Panel A shows autofluorescence of HS cell walls. C-J. Transverse sections of pZmSut1::RFP transgenic leaves located approximately half-way between the blade-sheath boundary and the base of the enclosing mature leaf (C, D), at the base of immature blade tissue (E, F), in immature sheath tissue (G, H), and located just above the meristem (I, J). Panels C, E, G, I, K, M show RFP images. Panels B, D, F, H, J, L, N show UV autofluorescence images. K, L. Close up of the lateral vein in middle of panels C, D. M, N. Close up of a developing lateral vein in panels E, F. Expression is observed in the protophloem (white arrow). Note with the RFP filter cube, at these exposure settings, virtually no red signal from chlorophyll is detected (cf. Fig. 4A, B). Scale bar in A-J = 100 μm . Scale bars in K-N = 25 μm .

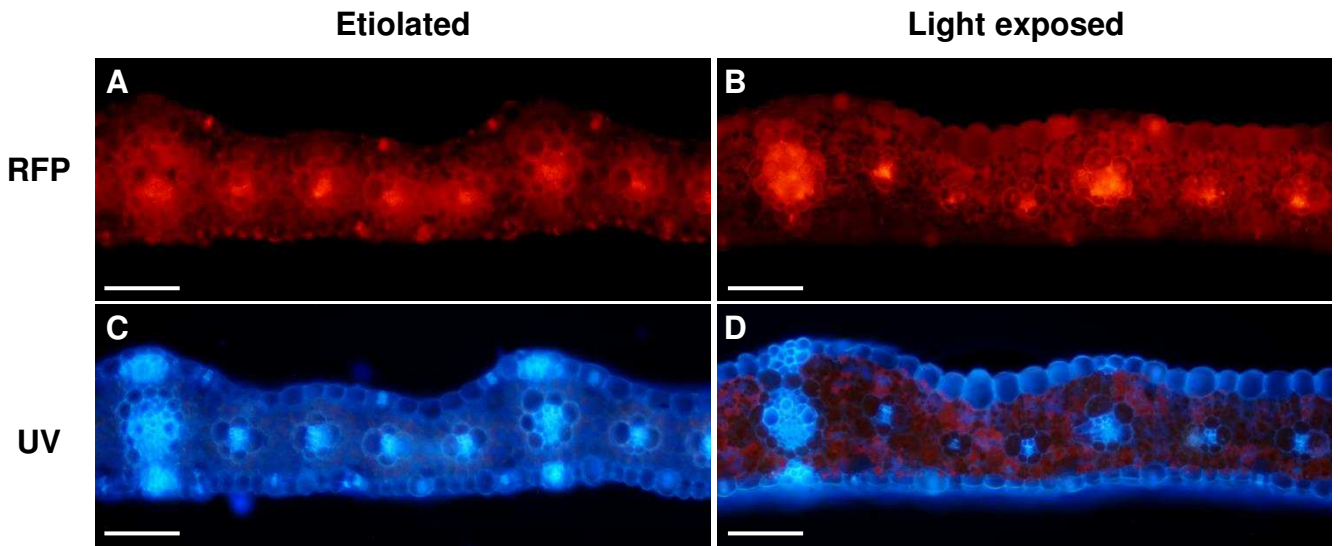


Figure 5. pZmSut1::RFP expression in leaves is induced in veins upon shifting plants from growth in the dark to the light. A, B. RFP images. C, D. UV autofluorescence images. A, C show an etiolated sink-leaf cross-section. B, D show a leaf cross-section after shifting plants into the light and the leaf matured as source tissue. Note with the RFP filter cube, at these exposure settings, virtually none of the red signal is from chlorophyll. Scale bar = 100 μ m.

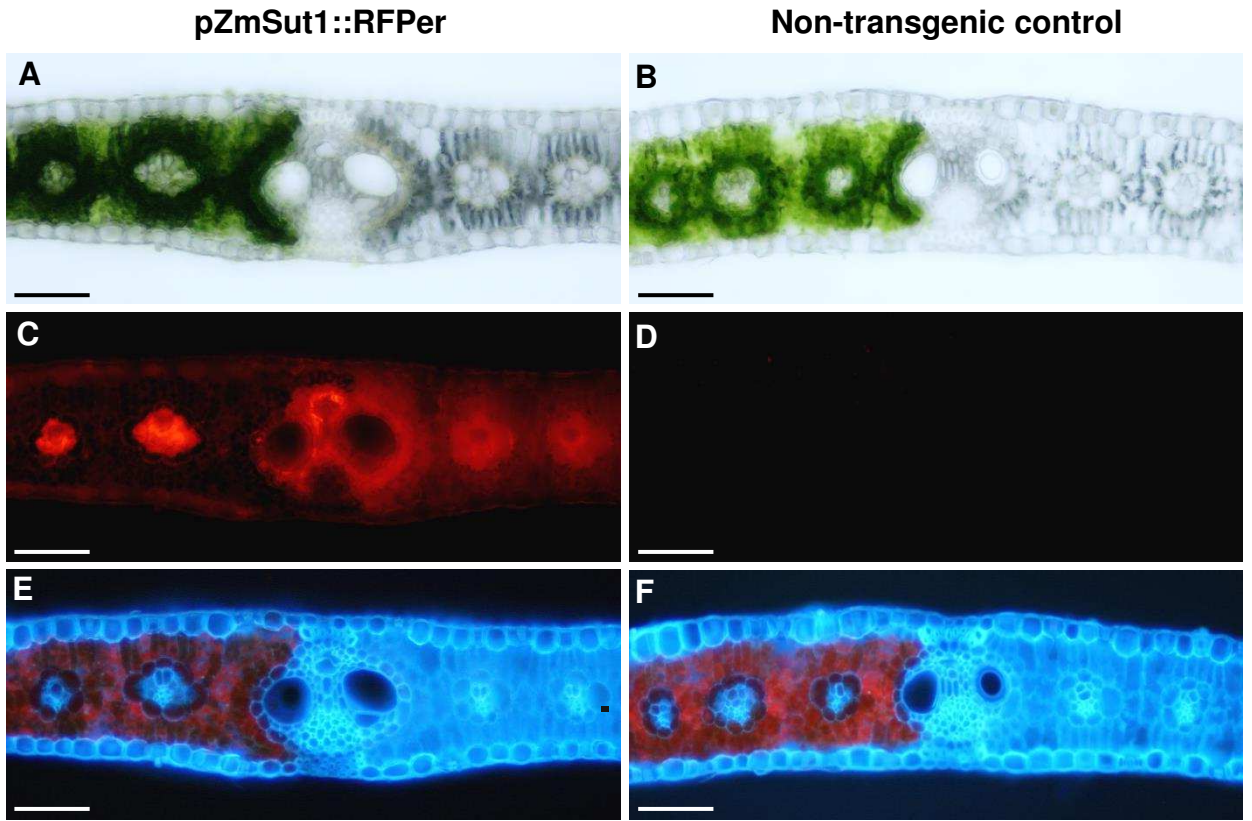


Figure 6. pZmSut1::RFP expression is induced in veins of mature source tissue compared with albino sink tissue in variegated *sr2* mutant leaves. A, C, E show a cross-section through a green-white border of a *sr2* leaf expressing pZmSut1::RFP. B, D, F show a non-transgenic control variegated *sr2* mutant leaf. A, B. Bright-field. C, D. RFP signal. E, F. UV autofluorescence. Note with the RFP filter cube, at these exposure settings, virtually no red signal from chlorophyll is detected (cf. Fig. 6D, F). Scale bar = 100 μ m.

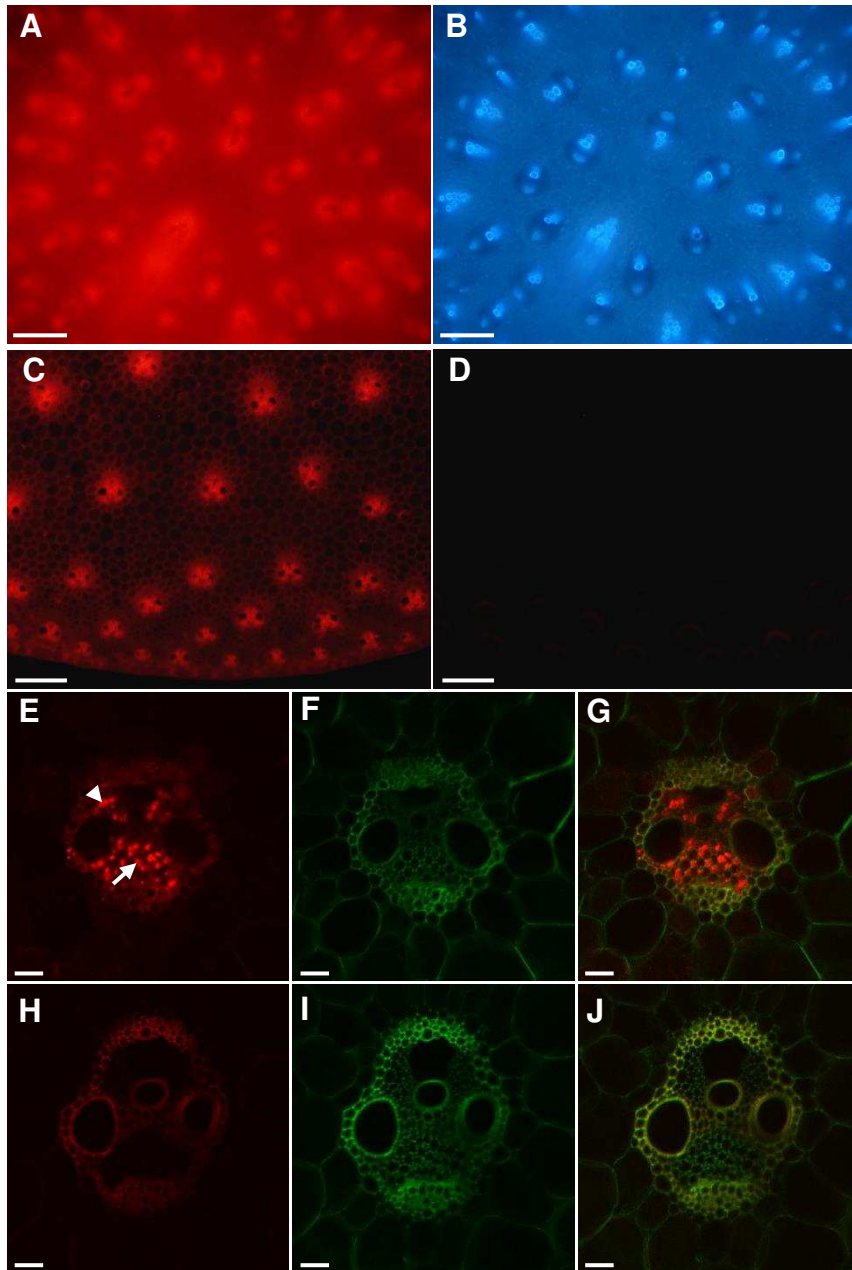


Figure 7. pZmSut1::RFP expression is broad initially in developing stem, but becomes restricted in mature stem veins. Transverse sections showing expression of the pZmSut1::RFP transgene in immature (A) and mature stem (C). B. UV autofluorescence of tissue shown in panel A. D. Transverse section of mature stem of non-transgenic control. A, C, D. RFP channel. A. Expression of the transgene is initially strongest in the protoxylem and protophloem, with lower signal in the developing parenchyma cells. C. At maturity pZmSut1::RFP expression is highest in veins, with low level in the storage parenchyma. E-G. Confocal images of mature stem vein showing pZmSut1::RFP expression in the XP cells (arrowhead) and CC (arrow). H-J. Confocal images of mature stem vein of non-transgenic control exhibiting autofluorescence. E, H. RFP channel. F, I. UV autofluorescence showing cell walls. G. Merged image of E and F. J. Merged image of H and I. Note with the RFP filter cube, at these exposure settings, virtually no red signal from chlorophyll is detected (cf. Fig. 7C, D). Scale bar in A, B = 250 μ m; in C, D = 500 μ m; in E-J = 25 μ m.

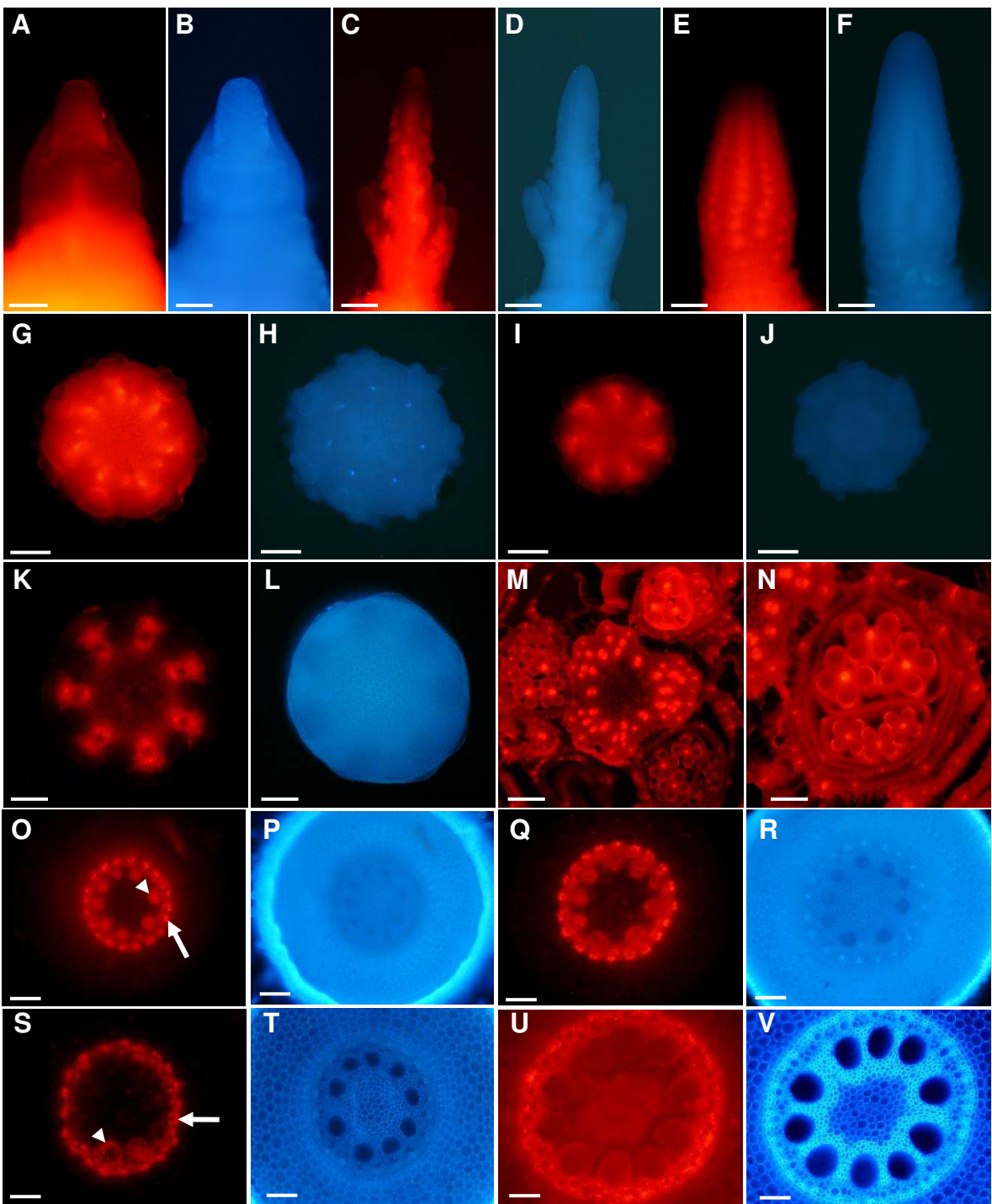


Figure 8. pZmSut1::RFP displays broad expression in multiple vegetative and reproductive sink tissues. Epi-fluorescent microscope images of pZmSut1::RFP expression in the shoot apical meristem, in developing tassels and ears, and in developing roots. A, C, E, G, I, K, M, N, O, Q, S, U. RFP signal. B, D, F, H, J, L, P, R, T, V. UV autofluorescence of corresponding tissue. A, B. Shoot apical meristem. C, D. Developing tassel. E-L. Developing ear. G-L represent cross-sections through the developing ear. M, N. Maturing tassel. O-V. Developing root. O. Transverse section near the root tip showing RFP expression is largely restricted to the phloem (arrow) and xylem (arrowhead). Q. Section slightly higher than that of O showing RFP expression in the phloem and developing xylem elements. S. Section at cusp between developing and mature xylem cells. Arrowhead indicates xylem element presumably undergoing autolysis. Arrow indicates phloem. U. Mature root. RFP expression can be seen in the phloem and diffusely throughout the root. Scale bar = 100 μ m for A, B, K, L, O-V; 250 μ m for C-J, N; 500 μ m for M. Exposure times for panels A, E, G, I, N = 750 ms; for C = 4 s; for K, M = 2 s. for O, Q, S, U = 1 s.

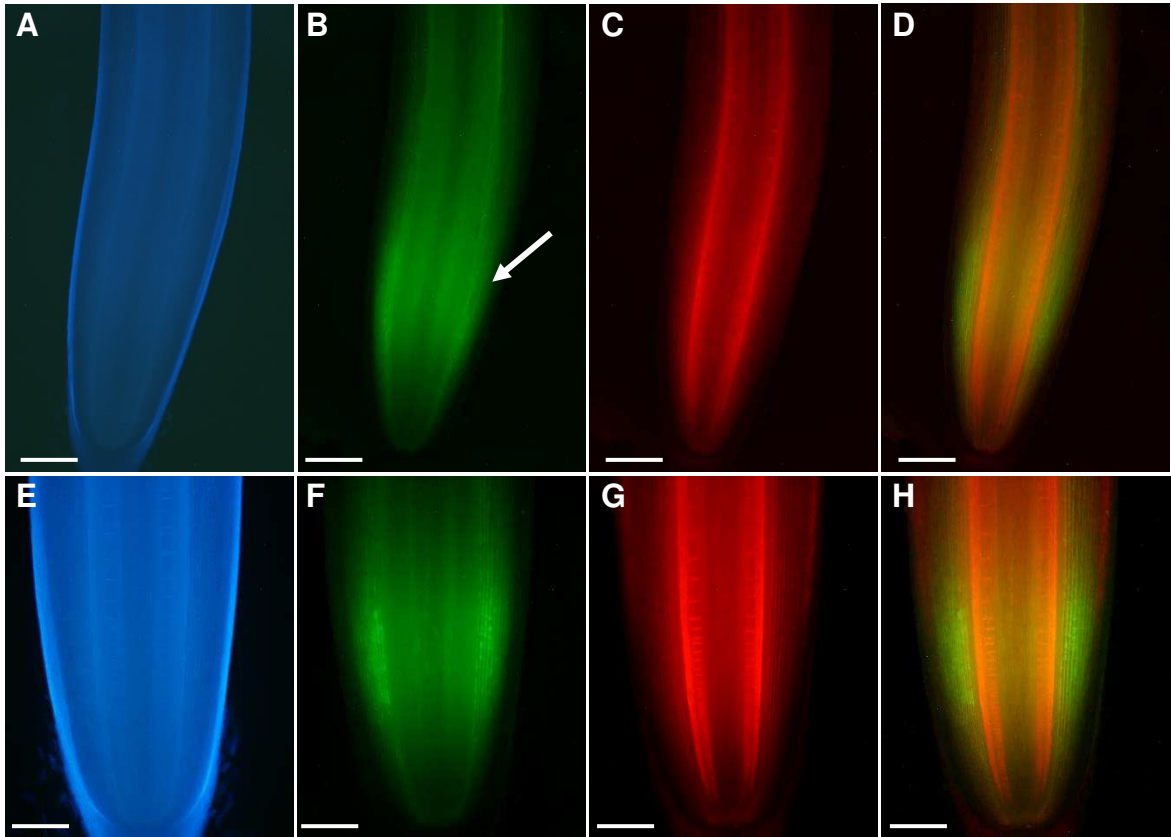


Figure 9. pZmSut1::RFP expression overlaps the phloem unloading zone identified by CF efflux into cortical cells of a pZmSut1::RFP transgenic root. A, E. UV autofluorescence. B, F. CF signal. White arrow indicates region of CF efflux from phloem into cortical cells. C, G. RFP signal. D, H. Overlay of the CF and RFP signals. E, F, G, and H are closer views of A, B, C, and D, respectively. Scale bar in A-D = 500 μ m; E-H = 250 μ m.

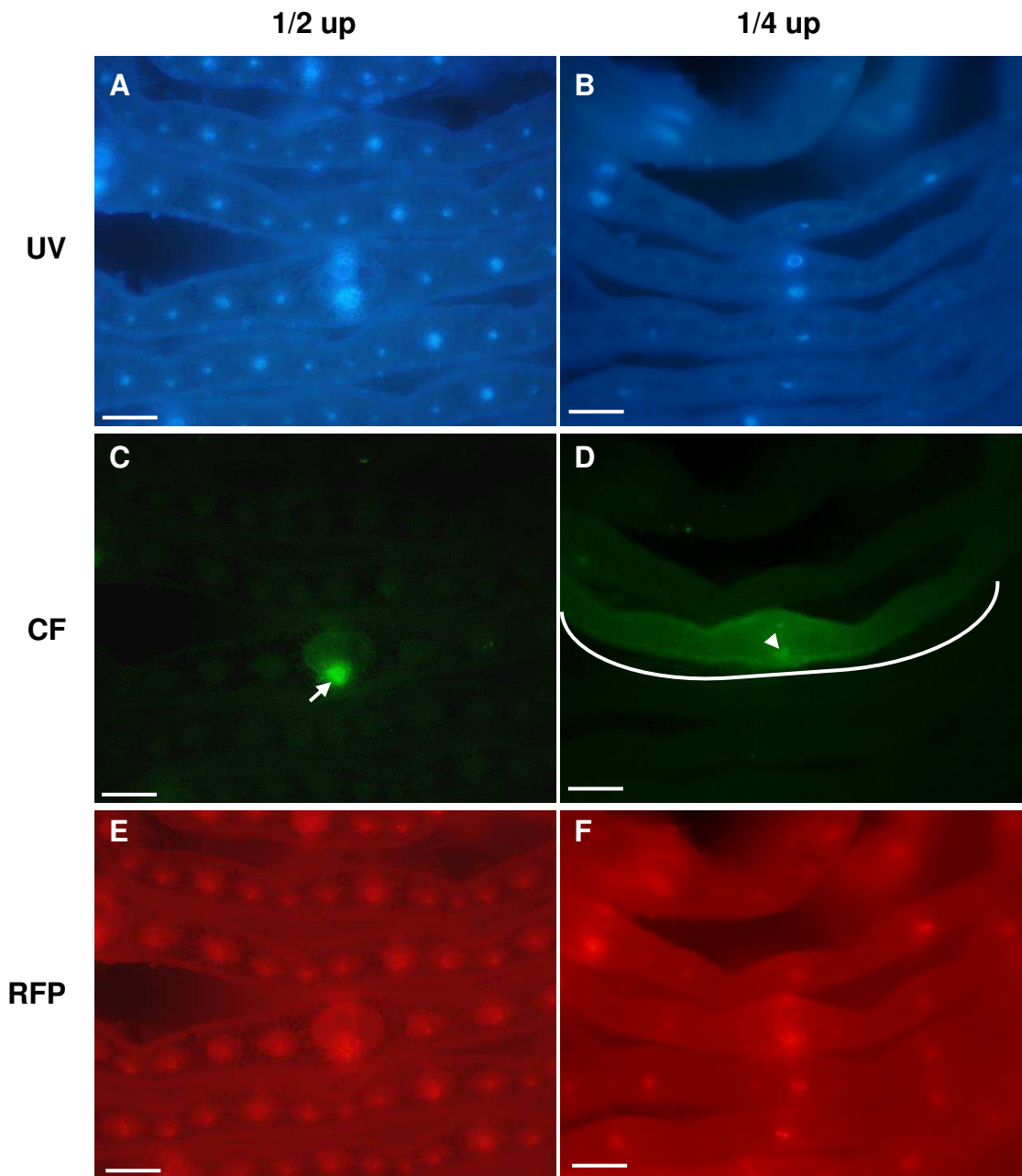


Figure 10. Developing leaves exhibit either symplasmic or apoplasmic phloem unloading in distinct regions that overlap pZmSut1::RFP_{er} expression. A, C, E show a cross-section taken approximately half-way up the blade of a developing pZmSut1::RFP_{er} expressing sink leaf. B, D, F show a cross-section taken approximately a quarter-way up the blade from the base of the same developing sink leaf. A, B. UV autofluorescence. C, D. CF signal. C. Arrow shows CF confinement within the symplasmically isolated phloem. D. Arrowhead shows vein symplasmically unloading CF into adjacent cells. CF movement marked by white bracket. E, F. RFP signal. Scale bar = 100 μ m.

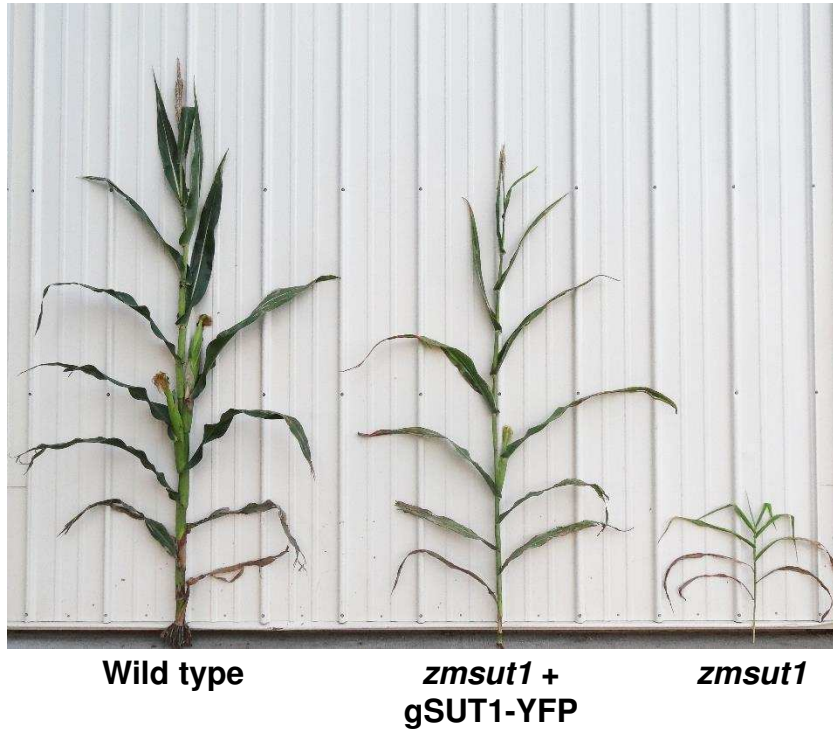


Figure 11. The gSut1-YFP transgene largely complements the *zmsut1* mutant phenotype. *zmsut1* homozygous mutant plants carrying the transgene (middle) grew to near wild-type height (left), and produced tassels that shed pollen and ears that produced silks. By contrast, the *zmsut1* homozygous mutants (right) that lacked the transgene and survived were stunted and typically failed to undergo anthesis or produce ears.

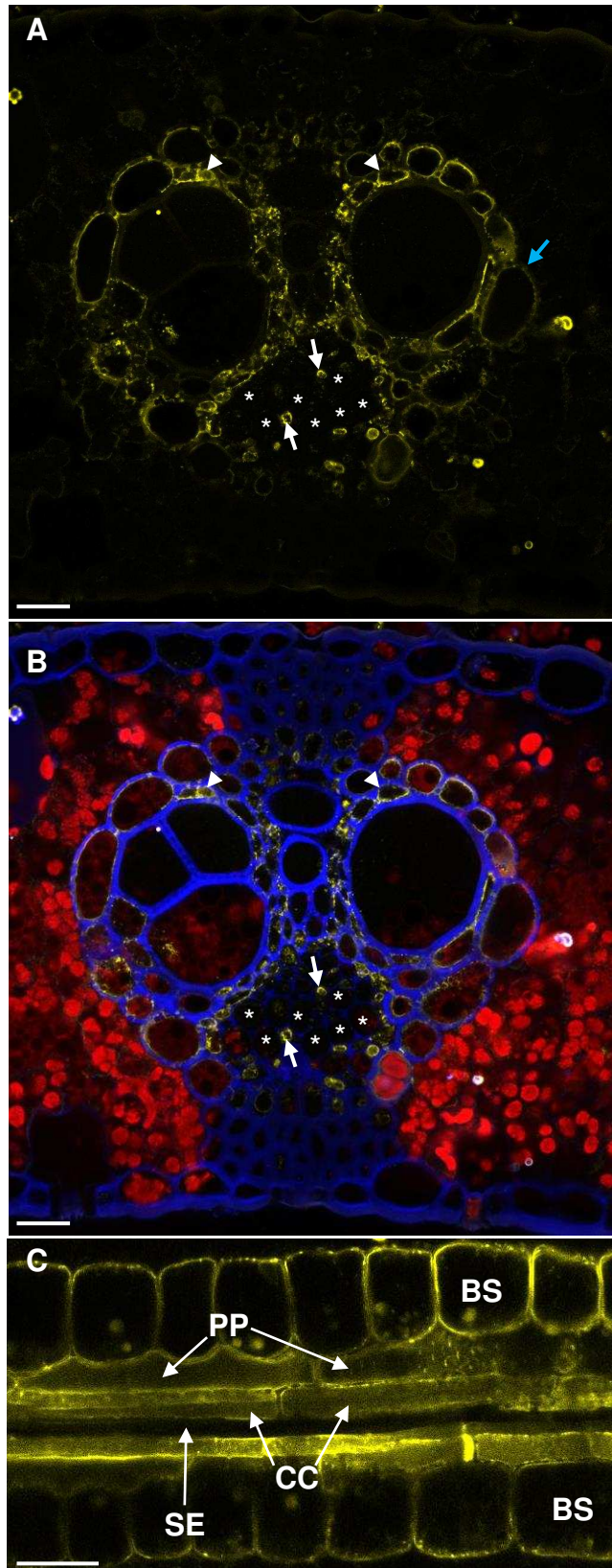


Figure 12. A ZmSUT1 protein translational fusion shows the same cellular expression pattern as observed with RNA in situ hybridization. Confocal images of the ZmSUT1 protein translationally fused at the C-terminus with YFP in transverse and paradermal leaf sections. A, B. Transverse section of a leaf lateral vein showing gSUT1-YFP is expressed in CC (arrows), XP (arrowheads), PP, and BS cells (blue arrow). Asterisks indicate SE. C. Paradermal section of a leaf minor vein showing gSUT1-YFP expression. A,C. YFP signal. B. Combined YFP, cell wall autofluorescence in blue, and chlorophyll autofluorescence in red. BS, bundle sheath cells, CC, companion cells, PP, phloem parenchyma cells, SE, sieve element. Scale bar = 25 μm .

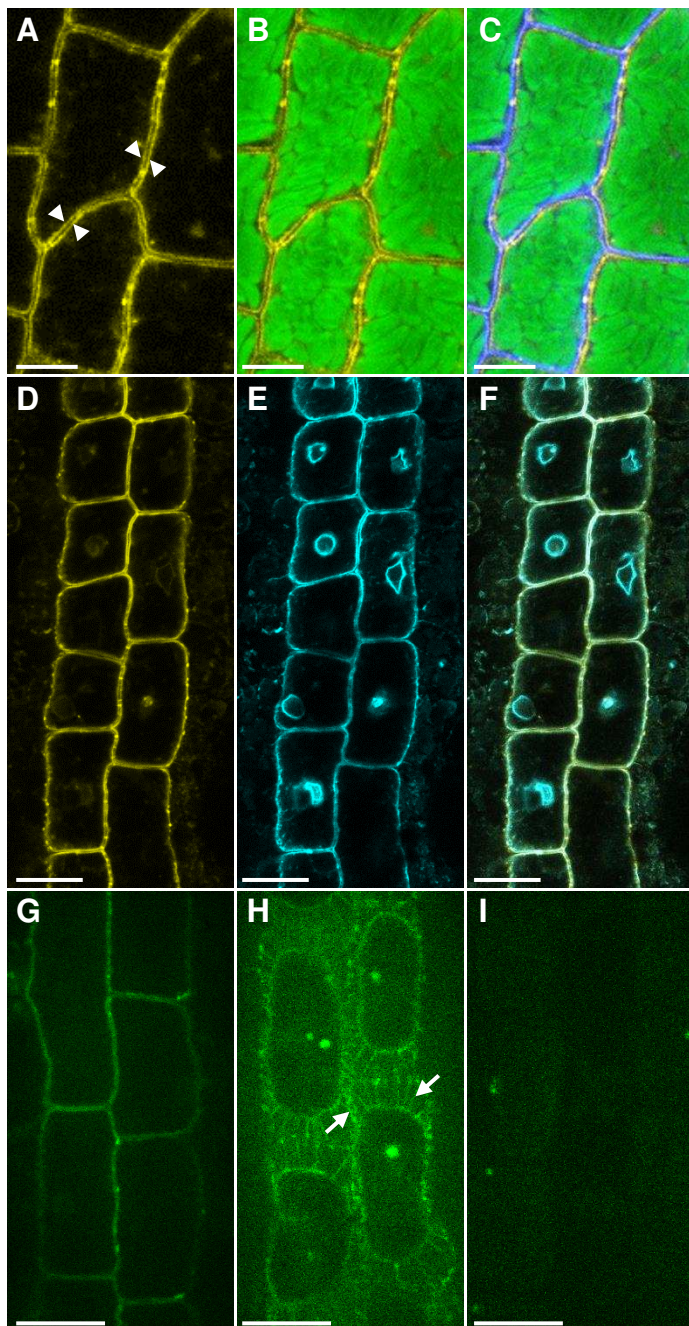


Figure 13. ZmSUT1 localizes to the plasma membrane. Confocal images of the expression of the SUT1 protein fused at the C-terminus with YFP. A-C. Paradermal section of a leaf minor vein focused on the BS cells. A. YFP signal. Arrowheads indicate YFP localization in two adjacent cells, separated by their shared cell wall. B. Combined YFP and chloroplasts (green). C. Combined YFP, chloroplasts, and cell wall (blue). D-F. Paradermal section of a leaf minor vein focused on the BS cells of a gSUT1-YFP and PIP2-1-CFP transgenic plant. D. YFP signal. E. CFP signal. F. Combined YFP and CFP signal. G-I. Spinning disc confocal image supporting ZmSUT1-YFP plasma membrane localization. G. Pre-plasmolysis YFP signal located at cell periphery. H. After plasmolysis with 0.75 M NaCl the plasma membrane has retracted; however, the plasma membrane is attached to the cell wall at the PD, resulting in the Hechtian strands (arrows). I. Non-transgenic control section. Scale bar = 10 μm for A-C, = 25 μm for D-I.

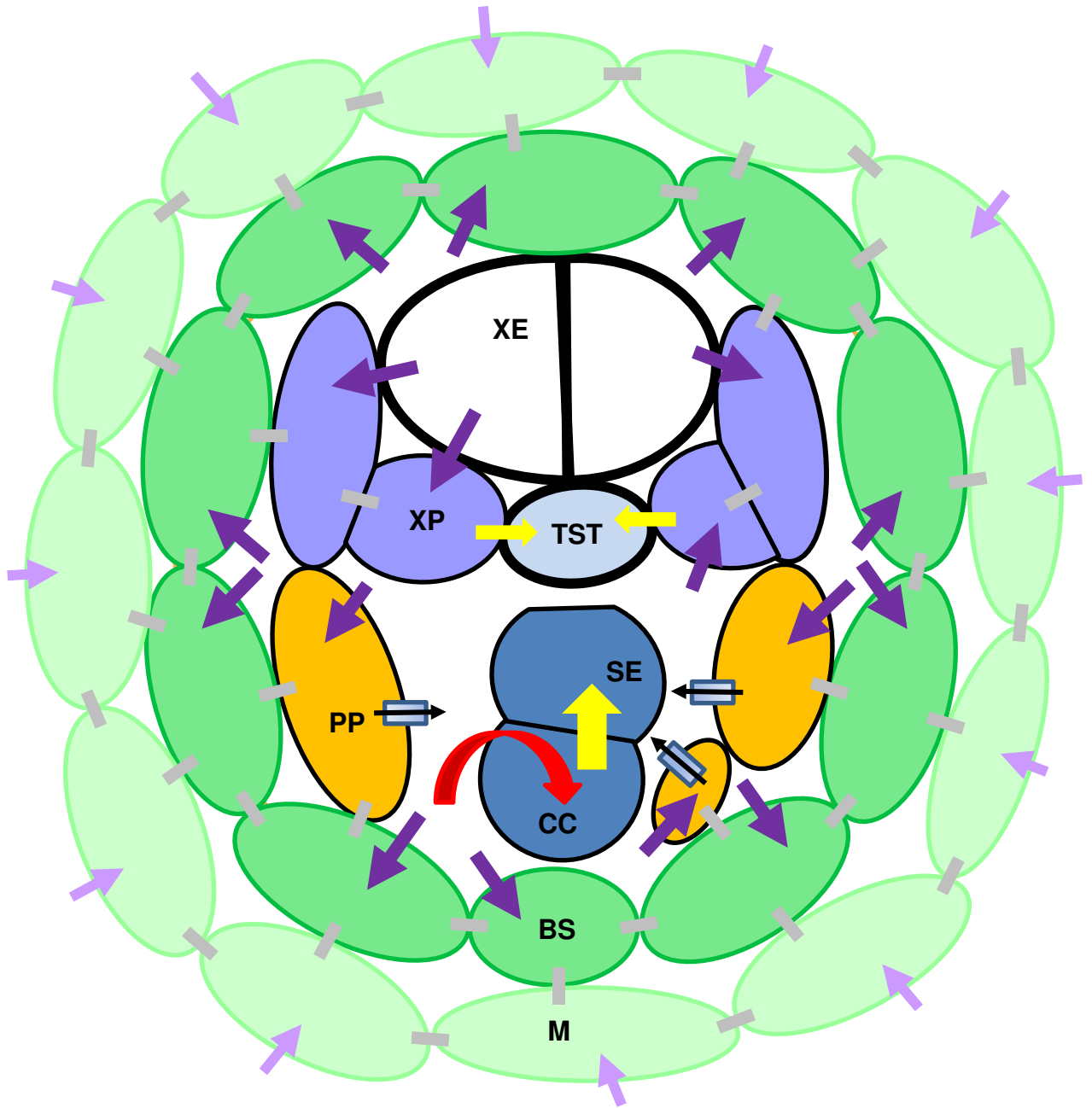


Figure 14. Model for dual functions of ZmSut1 in phloem loading and retrieval. Red arrow indicates ZmSUT1 loading sucrose into CC. Dark purple arrows indicate ZmSUT1 retrieving sucrose into non-conducting vascular cells; light purple arrows show ZmSUT1 recovering sucrose from leaf apoplast into M cells. Yellow arrows show symplasmic sucrose movement. Grey rectangles represent symplasmic connectivity through PD. Light blue rectangles with black arrows represent SWEET proteins effluxing sucrose to the apoplast of PP cells. Beige color represents vein apoplast. BS, bundle sheath cell, CC, companion cell, M, mesophyll cell, PP, phloem parenchyma cell, SE, sieve element, TST, thick-walled sieve element, XE, xylem element, XP, xylem parenchyma cell.

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