FLOWERING LOCUS C integrates carbon and nitrogen signaling for the proper timing of flowering in Arabidopsis

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Short title: C and N signaling regulate FLC.

Abstract

The timing of flowering in plants is modulated by both carbon (C) and nitrogen (N) signaling pathways. In a previous study, we established a pivotal role of the sucrose-signaling trehalose 6-phosphate pathway in regulating flowering under N-limited short-day conditions. In this work, we expand on our finding that wild-type plants grown under N-limited short days require an active trehalose 6-phosphate pathway to be able to flower. Both wild-type plants grown under N-limited conditions and knock-down plants of *TREHALOSE PHOSPHATE SYNTHASE1* induce *FLOWERING LOCUS C* expression, a well-known floral repressor associated with the vernalization response. When exposed to an extended period of cold, a mutant of *FLOWERING LOCUS C* fails to respond to N availability, and flowers at the same time under N-limited and full-nutrition conditions. Our data suggest that SUCROSE NON-FERMENTING 1 RELATED KINASE 1-dependent trehalose 6-phosphate-mediated C signaling and a novel mechanism downstream of N signaling likely involving NIN-LIKE PROTEIN 7 impact the expression of *FLOWERING LOCUS C.* Collectively, our data underscore the existence of a multi-factor regulatory system in which both C and N signaling pathways jointly govern the regulation of flowering in plants.

Keywords: TREHALOSE PHOSPHATE SYNTHASE (TPS1), Trehalose 6-phosphate (T6P), FLOWERING LOCUS C (FLC), nitrogen, N signaling, flowering time

52 **Introduction**

53 Owing to their sessile nature, plants adapt to environmental changes by
54 modifying their development and growth. These processes require significant
55 amounts of energy. Plants are in constant feedback with the environment and
56 their nutrient status, especially carbon (C) and nitrogen (N), that serve as crucial
57 bases for energy production and biomass generation. Low levels of C or N in
58 the cells suppresses development and growth in plants and triggers the onset
59 of senescence. To balance energy-intensive developmental processes with
60 endogenous nutrient availability, plants have evolved intricate signaling
61 networks (Fernie et al., 2020).

62 Flowering is an important developmental process in the life cycle of plants with
63 correct timing being essential for reproductive success. It is regulated by a
64 sophisticated genetic network that integrates various environmental and
65 endogenous signals to regulate the expression of the floral integrator genes
66 such as the florigen, *FLOWERING LOCUS T* (*FT*), and *SUPPRESSOR OF*
67 *OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Srikanth and Schmid, 2011;
68 Romera-Branchat et al., 2014; Song et al., 2015). FT integrates signals
69 perceived in the leaves and conveys this information to the shoot apical
70 meristem (SAM) to induce flowering (Corbesier et al., 2007; Jaeger and Wigge,
71 2007; Mathieu et al., 2007). At the SAM, FT interacts with the bZIP transcription
72 factor FLOWERING LOCUS D (FD) to form a complex that directly activates
73 *SOC1* along with floral meristem identity genes such as *APETALA1* (*AP1*) (Abe
74 et al., 2005; Wigge et al., 2005).

75 In addition to other stimuli, temperature impacts greatly the time of flowering.
76 Increased ambient temperature results in earlier flowering due to decreased
77 SVP protein stability (Lee et al., 2013; Lee et al., 2014). SVP forms a
78 temperature-dependent flowering repressor complex with partners such as
79 FLOWERING LOCUS M (FLM)/MADS AFFECTING FLOWERING1 (MAF1),
80 an orthologue of FLOWERING LOCUS C (FLC) (Pose et al., 2013;
81 Sureshkumar et al., 2016), resulting in earlier flowering when plants are
82 exposed to warmer conditions (Pose et al., 2013). SVP was also shown to
83 interact with FLC in a flowering repressor complex (Fujiwara et al., 2008; Li et
84 al., 2008). This delays floral transition by directly reducing the expression of *FT*,

85 *FD,* and *SOC1* (Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006;
86 Lee et al., 2007; Li et al., 2008). In winter-annual accessions of *Arabidopsis*
87 *thaliana* (Arabidopsis) flowering is suppressed due to active *FRIGIDA* (*FRI*)
88 resulting in promoted expression of *FLC*, unless the plants are exposed to a
89 long period of cold (vernalization process) (Sheldon et al., 2000). This
90 regulation involves a plethora of proteins and complexes acting in many layers
91 of gene regulation, ranging from RNA structures, epigenetic modification to
92 transcriptional and mRNA processing control (reviewed in Whittaker and Dean,
93 2017; and Sharma et al., 2020; Xu et al., 2021; Xu et al., 2021; Yang et al.,
94 2022).

95 Organic C and N supply is essential in particular for vegetative growth and plant
96 development (Sulpice et al., 2013). It is known that nutrients are essential for
97 developmental transitions (Fernie et al., 2020), but the underlying mechanisms
98 continue to be subject to active investigation. Interestingly, *FLC* expression was
99 observed to increase significantly in NITRATE TRANSPORTER 1.1 (NRT1.1)
100 and NRT1.13 defective mutant plants (Teng et al., 2019; Chen et al., 2021).
101 While NRT1.13 is suggested to be a nitrate transporter, NRT1.1 is a key
102 component of nitrate signaling functioning as both a transporter and a sensor
103 in roots (Li et al., 2021). This suggests a nitrate signaling-dependent control of
104 *FLC* as proposed by Kant and colleagues (Kant et al., 2011). This is supported
105 by the introduction of an *flc-3* mutation into the late-flowering NRT1.1 deficient
106 plant background which restored wild-type flowering (Teng et al., 2019).

107 Previous studies have identified multiple factors that influence N-regulated
108 flowering, which often vary and depend on the cultivation systems used (Lin
109 and Tsay, 2017). We are using a soil-based N-limited system developed by
110 (Tschoep et al., 2009), which allows plant adaptation and the investigation of
111 flowering time without stress-related symptoms (Olas et al., 2019; Olas et al.,
112 2021). With this system we previously reported that nitrate-regulated flowering
113 depends on SAM factors. Notably, in N-limiting conditions, nitrate-responsive
114 gene expression is affected and nitrate assimilation is reduced in the SAM (Olas
115 et al., 2019). The early nitrate response involves the NIN-LIKE PROTEIN (NLP)
116 transcription factors NLP6 and NLP7. They accumulate in the nucleus in the
117 presence of nitrate, regulating gene expression through nitrate responsive cis-

118 elements (NRE) (Konishi and Yanagisawa, 2013; Marchive et al., 2013).
119 Limited nitrate availability delays flowering due to decreased expression of
120 *SOC1*, likely through NLP6/NLP7-regulated expression of the SQUAMOSA
121 PROMOTER-BINDING PROTEIN-LIKE transcription factors encoding genes
122 *SPL3* and *SPL5* (Olas et al., 2019).
123 The sucrose signal trehalose 6-phosphate (T6P) regulates a plethora of
124 developmental and physiological responses (reviewed in Fichtner and Lunn,
125 2021). In Arabidopsis, T6P is synthesized by TREHALOSE PHOSPHATE
126 SYNTHASE1 (TPS1) (Vandesteene et al., 2010; Yang et al., 2012) and it acts
127 mainly by modulating the SUCROSE NON-FERMENTING 1 RELATED
128 KINASE 1 (SnRK1) activity. Moreover, T6P was suggested to be able to bind
129 directly to the SnRK1 upstream activating kinases and inhibit their activity (Zhai
130 et al., 2018). SnRK1 is a key sensor of energy status and it is required for both
131 normal growth and plant responses to stresses that impact plant fitness and
132 survival (Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008).
133 Although single mutants of SnRK1 catalytic subunits resemble wild-type plants
134 (Baena-Gonzalez et al., 2007; Jeong et al., 2015), *tps1* mutants (*tps1-2*) are
135 embryo-lethal (Eastmond et al., 2002). This can be bypassed by ectopically
136 expressing dexamethasone-inducible *TPS1* (*GVG::TPS1*) during seed set (van
137 Dijken et al., 2004). However, plants grown from these seeds remain in the
138 vegetative phase for a highly extended period or fail to flower entirely (van
139 Dijken et al., 2004; Wahl et al., 2013). T6P signaling induces flowering in leaves
140 via *FT* and also acts at the SAM through microRNA156 (miR156) and its target
141 transcripts, *SPL3-5* (Wahl et al., 2013), at least partially via the modulation of
142 the SUCROSE NON-FERMENTING 1 RELATED KINASE 1 (SnRK1) complex
143 activity (Zacharaki et al., 2022). This was supported by the observation that loss
144 of SnRK1 activity in the *tps1-2,GVG::TPS1* plants led to early induction of *FT*
145 in the leaves, reduced *miR156* levels and strong induction of *SPL3* in the SAM
146 during bolting (Zacharaki et al., 2022). Taken together these findings indicate
147 that both C and N signaling can target the same components of the flowering
148 network at the SAM (Wahl et al., 2013; Olas et al., 2019; Zacharaki et al., 2022),
149 underscoring their joint importance for the proper timing of flowering.
150 Even though the current understanding implies a straightforward output
151 downstream of nutrient signaling, our data now indicate a more complex

152 relationship between nutrient signaling and developmental programs. Here, we
153 demonstrate that the T6P pathway, which controls flowering under N limitation
154 in short days (Olas et al., 2019), impacts on the expression of *FLC* in addition
155 to *FLC* being differentially expressed upon exposure to contrasting N levels.
156 Our findings suggest that both C- and N-dependent pathways regulate
157 Arabidopsis flowering time by modulating *FLC* expression, implying a role in
158 the composition and timing of the FLC-SVP repressor complex within a
159 developmental context.
160

161 **Results**

162 **Sucrose signaling represses *FLC***

163 We have previously reported that plants grown under N-limited conditions and
164 short days (SD) accumulate both sucrose and T6P towards the end of the
165 vegetative growth phase. Importantly, *TPS1* knock-down plants
166 (*35S::amiRTPS1*) did not flower under these conditions (Olas et al., 2019). To
167 understand this phenomenon, we analyzed a developmental series of rosette
168 samples from both Col-0 and *35S::amiRTPS1* plants, focusing on candidate
169 genes, which specifically change their expression before the floral transition.
170 This analysis included multiple flowering time genes assessed by RT-qPCR.
171 Notably, we found a strong up-regulation of *FLC* expression in 4- to 6-days-old
172 and *MAF5* (*MADS AFFECTING FLOWERING 5*) expression in 12-days-old
173 *35S::amiRTPS1* plants (Fig. 1A, Fig. S1), similar to Zeng et al. (2024).
174 Considering that *FLC* expression has previously been suggested to be
175 modulated in response to N availability (Kant et al., 2011), it is an interesting
176 candidate for further investigation. We observed that *FLC* expression declines
177 before the floral transition, which occurs at 10 days after germination (DAG) in
178 Col-0 wild-type plants and 19 DAG in *35S::amiRTPS1* grown in full nutrition soil
179 (Wahl et al., 2013). This suggests that the T6P pathway fundamentally
180 contributes to the full repression of *FLC* in young seedlings. While we initially
181 did not anticipate that the T6P pathway could affect *FLC* expression at later
182 stages, we found that when the *flc-3* mutation is introduced into the *tps1-2*
183 *GVG::TPS1* background, it partially rescues the late flowering and the delayed
184 vegetative phase transition observed in this *tps1-2 GVG::TPS1* (Fig. 1B,C; Fig.
185 S2; Table S1,2). Our data, therefore, suggest that the T6P pathway is involved
186 in *FLC* regulation to promote flowering and facilitate the vegetative phase
187 change.

188

189 **FLC integrates N-signaling into the flowering network**

190 It has been previously shown that flowering is delayed in wild-type plants grown
191 in the limited N (LN) soil (Olas et al., 2019). Furthermore, some data suggest
192 that *FLC* expression may be influenced by N availability (Kant et al., 2011).
193 Thus, we conducted experiments to investigate the potential regulation of *FLC*
194 expression by N status. We grew wild-type Col-0 plants in a soil-based growth

195 system (Tschoep et al., 2009), consisting of a soil with optimal N (ON) and one
196 with LN source. We observed elevated *FLC* expression levels in LN in both
197 rosettes and apices of Col-0 plants grown continuously in SD conditions (Fig.
198 2A, B), and in apices of plants that were initially grown in SD conditions and
199 subsequently transferred to LD conditions (Fig. 2C). Similarly, we found
200 upregulation of *MAF5* levels in rosettes of LN grown plants (Fig. S3).
201 Considering that the *MAF5* was found to act downstream of the T6P pathway,
202 this finding suggests a link between N and T6P signaling pathways. (Fig. S1),
203 *MAF5* was also found to be upregulated in response to N limitation (Fig. S3).
204 Next, to obtain information on the expression pattern at higher spatial
205 resolution, we used *FLC* as a probe and performed RNA *in situ* hybridization
206 (Fig. 2D). *FLC* transcript was detectable at the SAM and in young leaves of LN
207 grown plants, confirming our previous observations that limited N availability
208 enhances *FLC* expression in plants. This finding suggests that FLC plays a role
209 in the regulation of flowering time in response to N availability.
210 It is well established that exposure to low temperatures decreases *FLC*
211 expression in plants (Searle et al., 2006). For this reason, we grew wild-type
212 plants at 4°C in SD for 8 weeks, followed by a transfer to 22°C until flowering.
213 This treatment resulted in wild-type plants flowering at the same time in both N
214 regimes, suggesting that FLC contributes to the delayed flowering time
215 observed in plants grown in the LN soil (Fig. 3A; Table S1).

216 FLC is known to form a flowering repressor complex with SVP to suppress
217 *SOC1* at the SAM (Li et al., 2008). Unlike *FLC*, *SVP* was not differentially
218 expressed in either LN-grown plants or *TPS1* knock-down plants (Fig. S4; Fig.
219 S5). Importantly, neither *flc-3* nor *svp-32* mutant plants responded to the
220 reduced N content in the LN soil (Fig. 3B; Table S1), flowering at the same time
221 in ON and LN conditions. This indicates that both FLC and SVP play a role in
222 the N-dependent regulation of flowering time.

223

224 **N-signaling affects *FLC* via NLP7**

225 NLPs are key regulators of nitrate sensing and signaling, with NLP6 and NLP7
226 being two of the most well-characterized members of this family in *Arabidopsis*
227 (Fredes et al., 2019). In the presence of nitrate, NLP7 is retained in the nucleus

228 through phosphorylation, where it binds to NREs present in N-responsive
229 genes to promote their expression (Konishi and Yanagisawa, 2013).
230 Interestingly, we observed a significant reduction of *FLC* expression in the *nlp7-*
231 *1* mutant, indicating that an active NLP7 modulates *FLC* expression when N is
232 not limited (Fig. 4).
233 Since the *FLC* gene does not carry an NRE in its promoter, genomic or
234 downstream sequences, we expanded our analysis to include other flowering
235 time genes that regulate *FLC* (Table S3). Notably, *FRI*, a key regulator
236 upstream of *FLC,* has four putative NREs (Table S3). However, in the Col-0
237 background, the *FRI* locus encodes an inactive protein and therefore does not
238 influence *FLC*. In addition to *FRI*, other genomic loci encoding *FLC* regulators
239 were also found with putative NREs (Table S3), but their expression was
240 unaffected under N limited conditions (Fig. S6). This was also the case in
241 *35S::amiRTPS1* plants (Fig. S7). Taken together, this suggests that *FLC*
242 suppression involves an as yet unknown transcription factor(s), whose activity
243 is regulated by NLP7.
244

245 **Sucrose and N-signals interconnect at the level of *FLC* for coordinated**
246 **flowering time regulation**
247 We have previously demonstrated that the T6P pathway and sufficient nitrate
248 levels are necessary for floral induction in SD (Olas et al., 2019). The fact that
249 *35S::amiRTPS1* plants fail to flower when N is limited and that *FLC* expression
250 is modulated by N availability, prompted us to test whether *FLC* is a target of
251 both N signaling and the T6P pathway.
252 We observed that *FLC* transcription was elevated in rosettes of wild-type plants
253 grown under SD with limited N which was even more pronounced in
254 *35S::amiRTPS1* plants (Fig. 5A). This suggests an additive effect between N
255 signaling and the T6P pathway, both converging on the *SPL3-5* node at the
256 SAM (Wahl et al., 2013; Olas et al., 2019). To test whether *FLC* could be
257 regulated through SPL3-5, we measured its expression in *spl345* mutants (Xie
258 et al., 2020). However, *FLC* expression in rosette leaves of *spl345* mutants was
259 comparable to that of wild-type plants (Fig. S8A), indicating that both pathways
260 regulate *FLC* expression via another mechanism. Similarly to *FLC*, we did not

261 observe any difference in *SVP* expression in *spl345* compared to Col-0 plants
262 (Fig. S8B).
263 The T6P pathway is known to function by directly modulating SnRK1 activity
264 (Zhang et al., 2009). Loss of SnRK1 activity restores flowering of *tps1* mutants
265 in LD by initial induction of *FT* in the leaves and subsequent suppression of
266 miR156 followed by SPLs induction in the SAM (Zacharaki et al., 2022). Thus,
267 we tested whether *FLC* regulation in *tps1* mutants is also mediated by SnRK1.
268 We found that indeed *FLC* expression was increased in the *tps1 GVG::TPS1*
269 mutant where SnRK1 is fully active. Interestingly, introducing non-catalytically
270 active mutations in SnRK1 within the *tps1 GVG::TPS1* background restores
271 *FLC* expression to wild-type levels in both rosette leaves and apex tissue (Fig.
272 5B,C). The suppression of *FLC* in the double mutant is more pronounced in the
273 apex than the rosette leaves, underscoring the critical role of the T6P pathway
274 in controlling developmental transitions. Our data suggest that *FLC* expression
275 is regulated by both nitrate and sugar availability via NPLs and the T6P pathway
276 through the SnRK1 complex, respectively (Fig. 6).
277
278 **Discussion**
279 C and N are essential for plant growth and development and the ability of plants
280 to properly sense their availability is crucial due to their sessile nature. C in the
281 form of sucrose is produced via photosynthesis in the leaves while N can be
282 taken up in both inorganic forms, as nitrate and ammonia, or organic forms, as
283 amino acids.
284 In *Arabidopsis*, a key sugar sensor is the T6P pathway which functions via
285 SnRK1 activity. The T6P pathway has a key role in plants' developmental
286 transitions, such as flowering. So far, it has been shown that both *miR156* and
287 *FT* regulation in the SAM and leaves, respectively, are required for *tps1* plants
288 to complete their transition to flowering (Wahl et al., 2013; Ponnu et al., 2020).
289 Here we found that *FLC,* a repressor of flowering, is also regulated by the T6P
290 pathway (Fig. 1A) and that loss of functional *FLC* partially restores flowering in
291 *tps1* (Fig. 1B,C). Although, we do not expect that *FLC* regulation is the prime
292 target of the T6P pathway under normal growth conditions, it could represent
293 an additional mechanism to prevent flowering under non-optimal growth
294 conditions.

295 Plants experiencing a sudden shift to colder temperatures have increased
296 amounts sucrose previously proposed to serve as a freezing protectant and
297 concomitant rising T6P levels (reviewed in Stitt and Hurry, 2002; Carillo et al.,
298 2013). During long cold exposure, *FLC* is suppressed and this regulation
299 involves several mechanisms, ranging from RNA structures to epigenetic
300 control. *FLC* suppression allows induction of *FT* and *SOC1* and flowering to
301 commence (Whittaker and Dean, 2017). In this scenario, when plants
302 experience cooler temperatures, nutrients that provide plants with C and N, are
303 transported and stored to serve as a basis for rapid growth for when conditions
304 are optimal again or used and metabolically transformed into cryoprotectants
305 to protect the cells from freezing damage (Kaplan et al., 2007). Thus, in sub-
306 optimal growth conditions, the T6P pathway might contribute to the suppression
307 of *FLC* in response to the C status.
308 N availability is a key factor in the regulation of plants' developmental processes
309 and phase transitions including the timing of flowering (Klebs, 1913; Dickens
310 and Van Staden, 1988; Bernier et al., 1993; Olas et al., 2019). Arabidopsis
311 cultivated on synthetic substrates exhibit early flowering in response to reduced
312 N levels (Castro Marin et al., 2011; Kant et al., 2011; Liu et al., 2013).
313 Conversely, soil-grown plants subjected to N limitation flower later than those
314 cultivated in soil without N limitation, which we previously linked to the induction
315 of *SPL3* and *SPL5* by NLP6 and NLP7 (Olas et al., 2019). In this study we
316 discovered that this phenotype can additionally be explained by significantly
317 elevated levels of *FLC* under N limitation (Fig. 2A-D). Furthermore, we found
318 that flowering time in plants with suppressed *FLC* due to the vernalization
319 response or with a non-functional *flc-3* allele is independent of N availability
320 (Fig. 3A,B). These results demonstrate that despite the general belief that *FLC*
321 does not play a major role in the regulation of flowering time in rapid-cycling
322 accessions, such as Col-0, *FLC* is required for fine-tuning the timing of floral
323 transition downstream of N signaling. Similar to *flc-3*, *svp-32* mutants flower at
324 the same time in the ON and LN soils (Fig. 3B), suggesting a role of *SVP* in N-
325 dependent flowering time regulation. However, in contrast to *FLC*, *SVP* is not
326 differentially expressed in plants grown in ON and LN soil (Fig. S5). FLC and
327 SVP proteins form a flowering repressor complex that delays floral transition by
328 directly reducing the expression of *FT* and *SOC1* (Hepworth et al., 2002;

329 Helliwell et al., 2006; Lee et al., 2007; Li et al., 2008). Given that both functional
330 *FLC* and *SVP* loci are required for the adjustment of flowering time in response
331 to N availability, it is likely that the N signal is integrated at the level of the FLC-
332 SVP complex. In this scenario, the formation of the repressor complex would
333 be tuned by the adjustment of *FLC* expression downstream of N-signaling.
334 Several transcription factors that are transcriptionally responsive to the N status
335 have been identified as prime responsive genes to N availability (Vidal et al.,
336 2015). NLPs are transcription factors facilitating nitrate signaling in plants, with
337 NLP6 and NLP7 representing the master regulators and the two most studied
338 (Fredes et al., 2019). In the absence of nitrate, NLP7 localizes strictly to the
339 cytosol, while exposure to nitrate triggers its localization into the nucleus where
340 it binds directly to NREs of nitrate-regulated genes (Konishi and Yanagisawa,
341 2013; Marchive et al., 2013). Since NREs are not present in the *FLC* locus
342 (Table S3), it is unlikely to be directly controlled by NLPs. Other examples of
343 *FLC* regulation related to N availability, are the *nrt1.1* and *nrt1.13*, mutants of
344 the nitrate sensor and transporter NRT1.1 and transporter NRT1.13 (Teng et
345 al., 2019; Chen et al., 2021). Similar to our findings (Fig. S7), expression of
346 known upstream regulators of *FLC* was not changed in *nrt1.13*, suggesting that
347 NRT1.13 regulates *FLC* expression and flowering time independently of these
348 known pathways (Chen et al., 2021).
349 Interestingly, we found that *FLC* was significantly downregulated in the late
350 flowering *nlp7-1* and *nlp6-2 nlp7-1* mutants grown on standard soil (Fig. 4A,
351 Fig. S9) indicating that NLP7 plays a role in the modulation of *FLC* expression.
352 Given the fact that NLP7 was found to control most of the nitrate-related gene
353 response (Marchive et al., 2013; Alvarez et al., 2020), the *nlp7-1* mutant is
354 thought to mimic a low nitrate state. Hence, this result appears to contradict our
355 observation of *FLC* accumulation in LN-grown plants (Fig. 2A,B). This could be
356 explained by the presence of an unknown NLP-independent mechanism
357 responsible for *FLC* upregulation in LN conditions. However, it should be noted
358 that in contrast to the mutant background, functional NLP7 is still present in
359 wild-type plants exposed to limited N. Thus, *nlp7-1* might not entirely mimic the
360 low-nitrate state after all and the absence of a functional NLP7 likely leads to
361 compensation by other NLPs. Furthermore, NLP proteins contain a PB1
362 domain, which mediates protein-protein interactions influencing NLP activity

363 (Konishi and Yanagisawa, 2019). Given this, NLP7 might form a complex with
364 an unknown *FLC* repressor, thereby preventing its nuclear localization under
365 low-nitrate conditions. In the absence of NLP7 or when plants are grown under
366 optimal N conditions, this potential repressor would localize into the nucleus,
367 leading to a repression of *FLC* expression. It will be interesting to further dissect
368 the mechanisms of *FLC* regulation downstream of N-signaling in the future.
369 Our data demonstrate that both the T6P and N-signaling pathways possibly
370 affect *FLC* expression via different mechanisms. Previous studies have
371 demonstrated that both pathways act via the miR156/SPLs node (Wahl et al.,
372 2013; Olas et al., 2019; Ponnu et al., 2020; Zacharaki et al., 2022). In particular,
373 the expression of *SPL3* and *SPL5* is reduced in plants grown in N-limited
374 environment (Bi et al., 2007; Pant et al., 2009; Krapp et al., 2011; Liang et al.,
375 2012; Fischer et al., 2013), suggesting a role for the miR156/SPL3/5 module in
376 the regulation of flowering time when N is limited. Similarly, the T6P pathway
377 acts via miR156 downregulation and SPL3-5 upregulation to induce flowering
378 and the vegetative phase change (Wahl et al., 2013; Ponnu et al., 2020;
379 Zacharaki et al., 2022). Although both pathways converge on the miR156/SPLs
380 module, *FLC* regulation seems to be independent (Fig. S8).
381 T6P has a key role in promoting growth and development by suppressing
382 SnRK1 complex activity, via direct binding to the SnRK1 upstream kinases
383 (Zhai et al., 2018). In a previous study, it was shown that *FT* was induced in the
384 double *tps1-2 GVG::TPS1 kin10-5* and *tps1-2 GVG::TPS1 snf4* mutant as early
385 as in wild-type plants (Zacharaki et al., 2022). Although this early *FT* induction
386 promoted the floral transition in wild-type plants within a few days, this was not
387 the case in both double mutants. The elevated expression of *FLC* in rosette
388 leaves of these mutants (Fig. 5C) could thus at least partially explain this
389 phenomenon. *FLC* downregulation is directly correlated with early *FT*
390 upregulation previously observed in the double mutants (Zacharaki et al.,
391 2022). Interestingly, we observed that *FLC* was also downregulated in the
392 double mutants in the apex (Fig. 5B) with more striking differences later on,
393 coinciding with the timing of floral transition (Zacharaki et al., 2022). In addition,
394 ectopic *FLC* expression in the SAM has been associated with delayed flowering
395 and reduced *SOC1* and *FD* expression (Sheldon et al., 2002; Noh and
396 Amasino, 2003; Searle et al., 2006). This is also the case in *tps1-2 GVG::TPS1*,

397 while gene expression is restored in the double mutants (Fig. S10) (Zacharaki
398 et al., 2022). Our data combined with the findings of Zeng et al. (2024) suggest
399 that the regulation of SnRK1 activity is essential for T6P-dependent floral
400 induction, which has several modes of action throughout the floral network to
401 ensure that sufficient energy is available for this demanding developmental
402 transition. Finally, our findings shed further light on the multifactorial aspects of
403 C- and N-dependent regulation of flowering time.
404

405 **Material and Methods**

406 Plant material and growth conditions

407 *Arabidopsis thaliana* plants used for this study are of the Columbia (Col-0)
408 ecotype. Mutant and transgenic lines such as *flc-3, svp-32*, *35S::amiRTPS1*,
409 *tps1-2,GVG::TPS1*, *tps1-2,GVG::TPS1,kin10-5*, *tps1-2,GVG::TPS1,snf4-1*,
410 *nlp6-2*, *nlp7-1*, *nlp6-2,nlp7-1* and *spl345* were previously described (Michaels
411 and Amasino, 1999; Lee et al., 2007; Wahl et al., 2013; Olas et al., 2019; Xie
412 et al., 2020; Zacharaki et al., 2022). The *flc-3,tps1-2,GVG*::*TPS1* double mutant
413 lines were generated by crossing. Genotypes were confirmed by a genotyping
414 PCR using the oligonucleotides listed in Table S4.

415 Arabidopsis plants were grown in controlled growth chambers (Model E-36L,
416 Percival Scientific Inc., Perry, IA, USA) at 22°C in long-day (LD, 16h light/8h
417 dark) or short-day (SD, 8h dark/16h light) conditions. Light intensity was
418 approximately 160 µmol/m²s. Controlled induction of flowering was performed
419 by transferring the plants from non-inductive (SD) to inductive conditions (LD)
420 as described (Schmid et al., 2003).

421 A previously established, almost natural, soil-based N-limited growth system
422 consisting of ON and LN soil was used to grow plants (Tschoep et al., 2009).
423 Briefly, the growth system consists of two types of peat-based soil mixtures with
424 either an optimal level of N (ON, ~850 mg (N)/kg) or a limited level of N (LN,
425 ~40 mg (N)/kg). Soil mixtures were prepared as described (Olas et al., 2019).

426 Phenotypic analyses

427 Flowering time was defined as days to flowering (DTF), which describes the
428 days after germination to the day of bolting (inflorescence length 0.5cm), and
429 by the total number of leaves (TLN). At least 16 plants were used to determine
430 flowering time of each genotype. For vegetative phase change, juvenile leaf
431 numbers were recorded and the leaf shape was digitally documented as
432 described (Ponnu et al., 2020). A student's *t*-test was used to test the
433 significance of the phenotypic differences.

434 Reverse transcription quantitative PCR (RT-qPCR)

435 Sampling, RNA extraction and RT-qPCR analysis of *FLC* in the *tps1-*
436 *2,GVG::TPS1*, *tps1-2,GVG::TPS1,kin10-5* and *tps1-2,GVG::TPS1,snf4-1* were

437 performed as described (Zacharaki et al., 2022). RNA extraction and RT-qPCR
438 analyses of all the other genes were performed according to Wahl et al. (2013).
439 Relative expression values were calculated with the 2^DDCt method using Ct
440 values of a housekeeping gene index of *TUB2* (At5g62690), *SAND*
441 (At2g28390), *UBQ10* (At4g05320), and *PDF2* (At1g13320). RT-qPCR
442 analyses were performed in three or four biological replicates (n=3 or 4). A
443 Student's *t*-test was used to test for statistical significance.

444 RNA *in situ* hybridization

445 Wax embedding, sectioning, RNA *in situ* hybridization, and imaging were
446 performed as described (Wahl et al., 2013; Gramma and Wahl, 2023). Probes
447 were synthesized using the DIG RNA Labeling Kit (Roche, Mannheim,
448 Germany) for CDS of the *FLC* gene cloned into the pGEM®-T Easy vector
449 (Promega, Madison, Wisconsin, US). Oligonucleotides and construct IDs are
450 listed in Table S4.
451

452 **Accession numbers**

453 TPS1 (At1g78580), FLC (At5g10140), SVP (At2g22540), MAF5 (At5g65080),
454 FCA (At2g19520), EMF1 (At5g11530), PIE1/SNF2 (At3g12810), NLP6
455 (At1g64530), NLP7 (At4g24020), FRI (At4g00650), SUF4 (At1g30970), ELF7
456 (At1g79730), SEF (At5g37055), VRN1 (At3g18990), VRN2 (At4g16845),
457 EMF2/CYR1 (At5g51230), TFL2 (At5g17690), FVE (At2g19520), HUA2
458 (At2g19520), SNF4 (At1g09020), KIN10 (At3g01090), SPL3 (At2g33810),
459 SPL4 (At1g53160), SPL5 (At3g15270).
460

461 **Data availability**

462 The data supporting the findings of this study are included in this manuscript or
463 the supplemental information and material can be obtained from the
464 corresponding author upon reasonable request.

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Author's contribution

VW conceived and designed the experiments and prepared the figures. All authors performed essential experiments and analyzed data: JJO and VW performed the RNA *in situ* hybridizations; VG, JJO, VZ, and MML the RT-qPCRs; JJO, UML and JP performed phenotypic analyses. VG, VZ and VW wrote the manuscript with contributions from the other authors. All authors have read and commented on the text and figures within this manuscript.

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710 **Figure legends**

711

712 **Figure 1**. **The Trehalose 6-phosphate pathway impacts on *FLOWERING***
713 ***LOCUS C*.** (**A**) Expression of *FLOWERING LOCUS C* measured by RT-qPCR
714 in rosettes of Col-0 and *35S::amiRTPS1* plants grown under long days (16h
715 light/ 8h darkness). *n* = 4. (**B**) Flowering time measured as leaf numbers (rosette
716 leaves in gray; cauline leaves in white). n ≥ 15 individual plants per genotype.
717 (**C**) Representative photographs of the plants analyzed in (B). Abbreviations:
718 days after germination (DAG). Data represents mean, error bars are standard
719 deviations (s.d.), statistically significant difference compared to Col-0 wild-type
720 (Student *t*-test, **P*<0.05, ***P*<0.01, and ****P*<0.001).

721 **Figure 2**. ***FLOWERING LOCUS C* in response to nitrogen limitation.** (**A, B**)
722 Expression of *FLOWERING LOCUS C* measured by RT-qPCR in rosettes (A)
723 and apices (B) of Col-0 plants grown in optimal nitrogen (ON) and limited-
724 nitrogen (LN) conditions under short days (8h light/ 16h dark). (**C**) *FLOWERING*
725 *LOCUS C* expression measured by RT-qPCR in apices of plants initially grown
726 under short days (30 days) and then transferred to long days to initiate the floral
727 transition for 3, 5, and 7 days. (**D**) RNA *in situ* hybridization using *FLOWERING*
728 *LOCUS C* specific probe on longitudinal sections through vegetative apices of
729 Col-0 plants grown in ON and LN soils. Abbreviations: days after germination
730 (DAG); days after shift (DAS). Data represents mean, error bars are standard
731 deviations (s.d.), n=3, statistically significant difference between ON and LN
732 (Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001). Star indicates apex summit.

733 **Figure 3**. **FLOWERING LOCUS C and SHORT VEGETATIVE PHASE are**
734 **required for the limited nitrogen-dependent flowering response.** (**A**)
735 Flowering time of Col-0 wild-type plants treated with an 8-week period of cold.
736 Note that afterwards plants were transferred to 22°C until flowering. (**B**)
737 Flowering time of Col-0, *flc-3,* and *svp-32* mutant plants grown under short-day
738 (8h light/16h darkness) conditions. Data represents mean, error bars are
739 standard deviations (s.d.), n ≥ 15 individual plants per genotype, statistically
740 significant difference between ON and LN (Student's *t*-test, ****P*<0.001).

741 **Figure 4**. ***FLOWERING LOCUS C* expression downstream of NIN-LIKE**
742 **PROTEIN 6 (NLP6) and NIN-LIKE PROTEIN 7 (NLP7).** Expression of
743 *FLOWERING LOCUS C* measured by RT-qPCR at 10 days after germination
744 (DAG) in rosettes of Col-0, *nlp6-2,nlp7-1*, and *nlp6-2,nlp7-1* plants. Data
745 represents mean, error bars are standard deviations (s.d.), n=3, statistically
746 significant difference compared to Col-0 wild-type (Student's *t*-test, **P*<0.05,
747 ***P*<0.01).

748 **Figure 5**. **Trehalose 6-phosphate pathway and nitrogen-signaling**
749 **converge at *FLOWERING LOCUS C*.** (**A**, **B**, **C**) Expression of *FLOWERING*
750 *LOCUS C* measured by RT-qPCR in (A) rosettes of wild-type Col-0 and
751 *35S::amiRTPS1* plants grown in optimal nitrogen (ON) and limited-nitrogen
752 (LN) conditions under short days (8h light/ 16h dark) at 60 days after
753 germination (DAG), in (B) apices and (C) rosettes of wild-type Col-0, *tps1-*
754 *2,GVG:TPS1*, *snf4,tps1-2,GVG:TPS1* and *kin10,tps1-2,GVG:TPS1* plants
755 grown in standard soil under long days (16h light/ 8h dark). Data represents
756 mean, error bars are standard deviations (s.d.), statistically significant
757 difference compared to Col-0 wild-type (Student *t*-test, **P*<0.05, ***P*<0.01, and
758 ****P*<0.001).

759 **Figure 6**. **Carbon and nitrogen signaling target similar components of the**
760 **flowering network in the shoot apical meristem for the proper timing of**
761 **flowering**. *FLOWERING LOCUS C,* a key repressor of flowering, is not only
762 regulated by cold temperature as part of the vernalization process, but is also
763 affected by nutrient availability. The Trehalose 6-phosphate pathway negatively
764 impacts *FLOWERING LOCUS C* via SUCROSE NON-FERMENTING 1
765 RELATED KINASE 1. Nitrogen signaling controls *FLOWERING LOCUS C* via
766 a yet to identify mechanism involving NIN-LIKE PROTEIN 7. The repressor
767 complex composed of FLOWERING LOCUS C and SVP is eventually tuned by
768 the adjustment of *FLOWERING LOCUS C* expression downstream of both
769 carbon and nitrogen signaling to control *SUPPRESSOR OF*
770 *OVEREXPRESSION OF CONSTANS 1* in the shoot apical meristem.
771 Independently, both nutrient pathways work via the age pathway (SQUAMOSA
772 PROMOTER-BINDING PROTEIN-LIKE 3-5) to induce flowering.

773 **Supplemental Material**

Supplemental Figure S1. *MADS AFFECTING FLOWERING 5* in *35S::amiRTPS1* plants.

Supplemental Figure S2. *flc-3* partially suppresses the delayed vegetative phase change phenotype of *tps1-2,GVG::TPS1* plants.

Supplemental Figure S3. *MADS AFFECTING FLOWERING 5* in response to nitrogen limitation.

Supplemental Figure S4. *SHORT VEGETATIVE PHASE* in *35S::amiRTPS1* plants.

Supplemental Figure S5. *SHORT VEGETATIVE PHASE* in response to nitrogen limitation.

Supplemental Figure S6. Regulators upstream *FLOWERING LOCUS C* in response to N limitation.

Supplemental Figure S7. Regulators upstream *FLOWERING LOCUS C* in *35S::amiRTPS1* plants.

Supplemental Figure S8. *FLOWERING LOCUS C* and *SHORT VEGETATIVE PHASE* in *spl345* mutant plants.

Supplemental Figure S9. Plant phenotype of *nlp6* and *nlp7* mutant plants.

Supplemental Figure S10. *FLOWERING LOCUS D* in *snrk1,tps1-2,GVG::TPS1* mutants.

Supplemental Table S1. Flowering time data of experiments described in this study (Figure S2).

Supplemental Table S2. Vegetative phase change data of experiment described in this study

Supplemental Table S3. Putative nitrate responsive *cis*-elements (NREs) in regulators upstream *FLOWERING LOCUS C.*

Supplemental Table S4. Oligonucleotides used in this study.

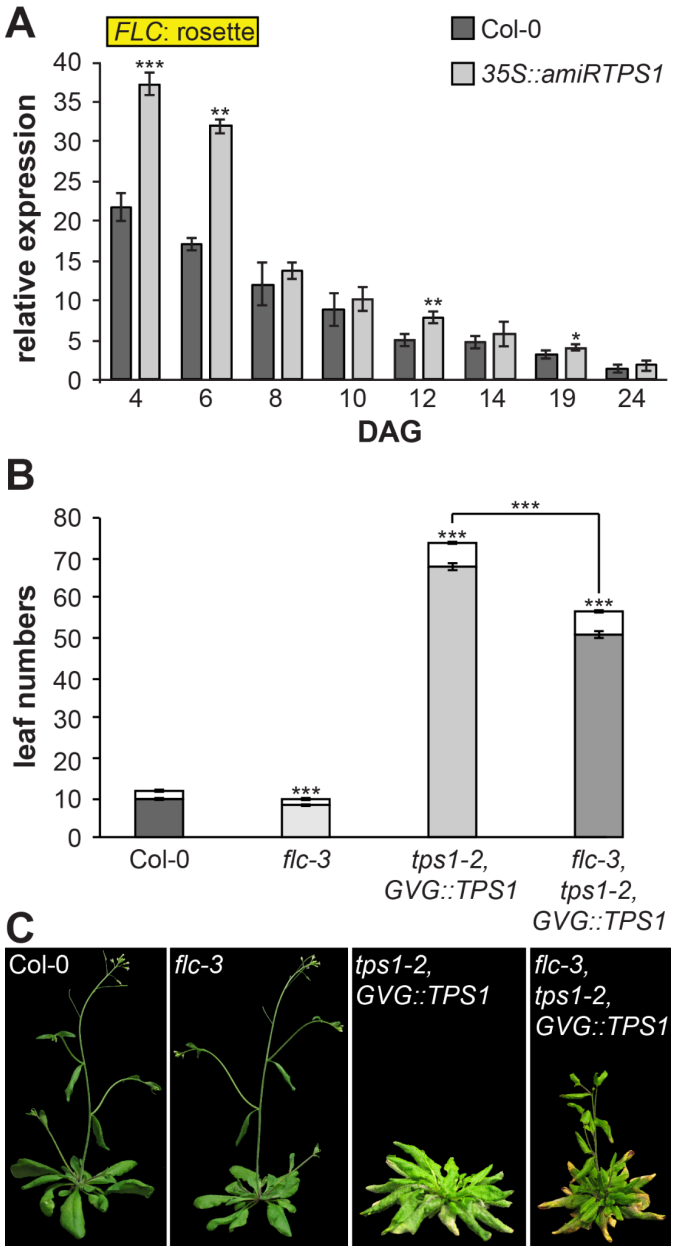

Figure 1. **The Trehalose 6-phosphate pathway impacts on *FLOWERING LOCUS C*.** (**A**) Expression of *FLOWERING LOCUS C* measured by RT-qPCR in rosettes of Col-0 and *35S::amiRTPS1* plants grown under long days (16h light/ 8h darkness). *n* = 4. (**B**) Flowering time measured as leaf numbers (rosette leaves in gray; cauline leaves in white). n ≥ 15 individual plants per genotype. (**C**) Representative photographs of the plants analyzed in (B). Abbreviations: days after germination (DAG). Data represents mean, error bars are standard deviations (s.d.), statistically significant difference compared to Col-0 wild-type (Student *t*-test, **P*<0.05, ***P*<0.01, and ****P*<0.001).

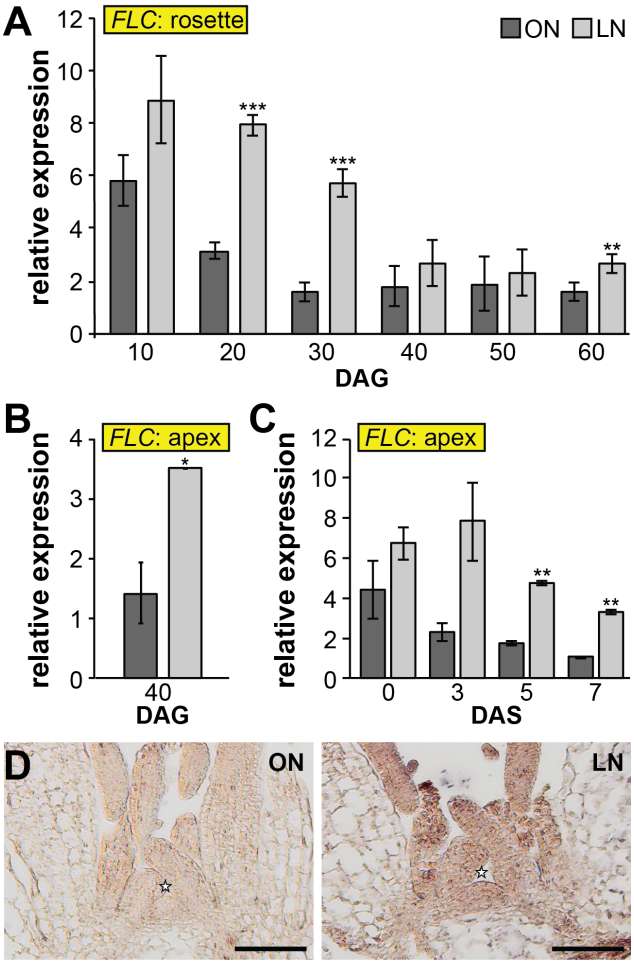

Figure 2. ***FLOWERING LOCUS C* in response to nitrogen limitation.** (**A, B**) Expression of *FLOWERING LOCUS C* measured by RT-qPCR in rosettes (A) and apices (B) of Col-0 plants grown in optimal nitrogen (ON) and limited-nitrogen (LN) conditions under short days (16h light/ 8h dark). (**C**) *FLC* expression measured by RT-qPCR in apices of plants initially grown under short days (30 days) and then transferred to long days to initiate the floral transition for 3, 5, and 7 days. (**D**) RNA *in situ* hybridization using *FLOWERING LOCUS C* specific probe on longitudinal sections through vegetative apices of Col-0 plants grown in ON and LN soils. Abbreviations: days after germination (DAG); days after shift (DAS). Data represents mean, error bars are standard deviations (s.d.), n=3, statistically significant difference between ON and LN (Student's *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001). Star indicates apex summit.

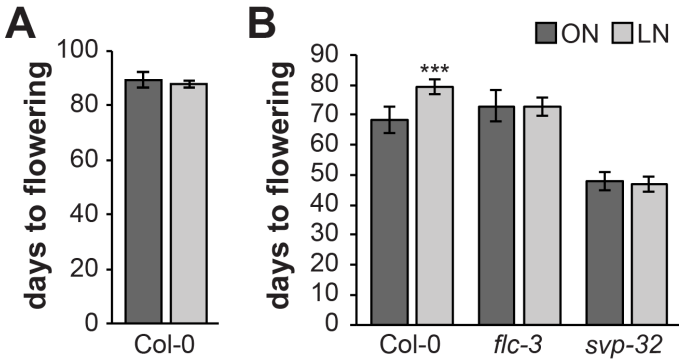

Figure 3. **FLOWERING LOCUS C and SHORT VEGETATIVE PHASE are required for the limited nitrogen-dependent flowering response.** (**A**) Flowering time of Col-0 wild-type plants treated with an 8-week period of cold. Note that afterwards plants were transferred to 22°C until flowering. (**B**) Flowering time of Col-0, *flc-3,* and *svp-32* mutant plants grown under short-day (8h light/16h darkness) conditions. Data represents mean, error bars are standard deviations (s.d.), n ≥ 15 individual plants per genotype, statistically significant difference between ON and LN (Student's *t*-test, ***$P<0.001$).

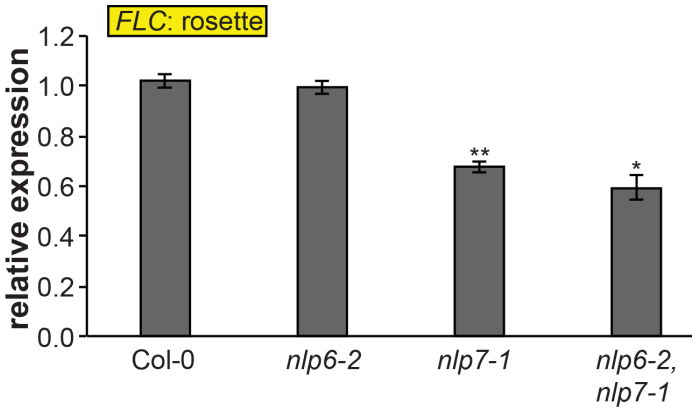

Figure 4. ***FLOWERING LOCUS C* expression downstream of NIN-LIKE PROTEIN 6 (NLP6) and NIN-LIKE PROTEIN 7 (NLP7).** Expression of *FLOWERING LOCUS C* measured by RT-qPCR at 10 days after germination (DAG) in rosettes of Col-0, *nlp6-2*, *nlp7-1*, and *nlp6-2,nlp7-1* plants. Data represents mean, error bars are standard deviations (s.d.), n=3, statistically significant difference compared to Col-0 wild-type (Student's *t*-test, **P*<0.05, ***P*<0.01).

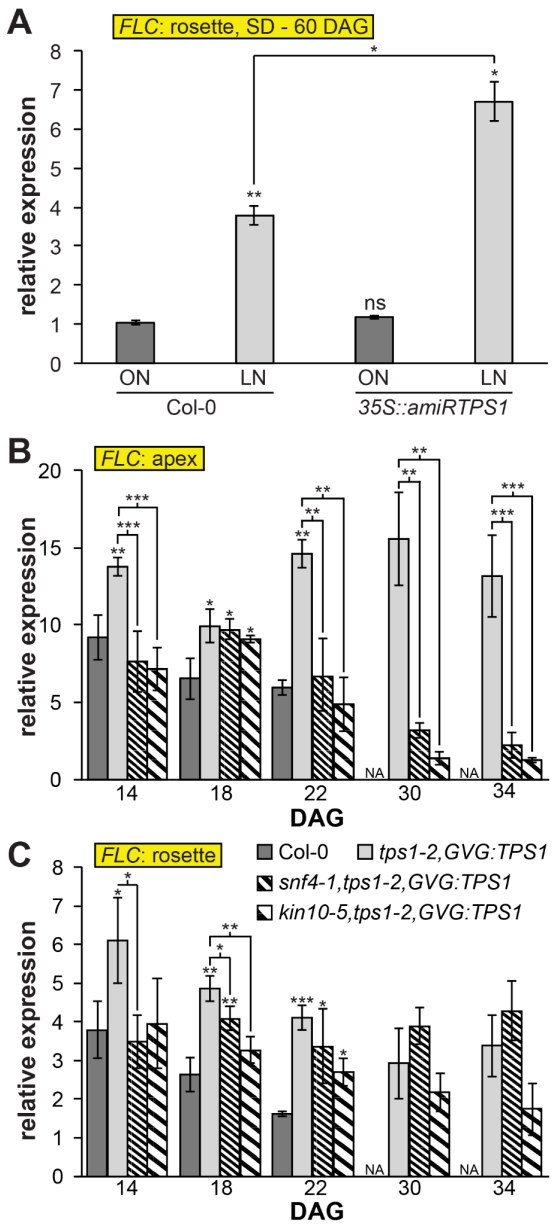

Figure 5. Trehalose 6-phosphate pathway and nitrogen-signaling converge at *FLOWERING LOCUS C*. (**A**, **B**, **C**) Expression of *FLOWERING LOCUS C* measured by RT-qPCR in (A) rosettes of wild-type Col-0 and *35S::amiRTPS1* plants grown in optimal nitrogen (ON) and limited-nitrogen (LN) conditions under short days (8h light/ 16h dark) at 60 days after germination (DAG), in (B) apices and (C) rosettes of wild-type Col-0, *tps1-2,GVG:TPS1*, *snf4,tps1-2,GVG:TPS1* and *kin10,tps1-2,GVG:TPS1* plants grown in standard soil under long days (16h light/ 8h dark). Data represents mean, error bars are standard deviations (s.d.), significant difference compared to Col-0 wild-type (Student *t*-test, **P*<0.05, ***P*<0.01, and ****P*<0.001).

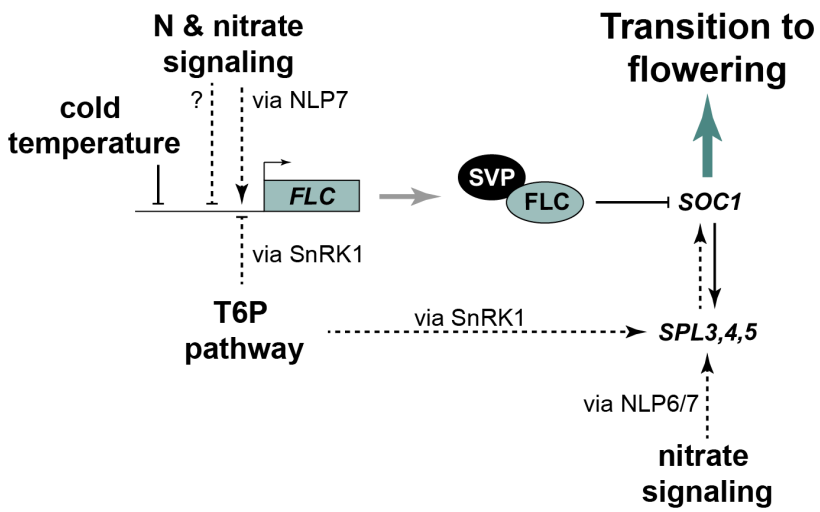

Figure 6. **Carbon and nitrogen signaling target similar components of the flowering network in the shoot apical meristem for the proper timing of flowering**. *FLOWERING LOCUS C,* a key repressor of flowering, is not only regulated by cold temperature as part of the vernalization process, but is also affected by nutrient availability. The Trehalose 6-phosphate pathway negatively impacts *FLOWERING LOCUS C* via SUCROSE NON-FERMENTING 1 RELATED KINASE 1. Nitrogen signaling controls *FLOWERING LOCUS C* via a yet to identify mechanism involving NIN-LIKE PROTEIN 7. The repressor complex composed of FLOWERING LOCUS C and SVP is eventually tuned by the adjustment of *FLOWERING LOCUS C* expression downstream of both carbon and nitrogen signaling to control *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* in the shoot apical meristem. Independently, both C and N pathways work via the age pathway (SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 3-5) to induce flowering.