

1 **Tudor staphylococcal nuclease acts as a docking platform for stress granule components**
2 **in *Arabidopsis thaliana***

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39 **SUMMARY**

40 Adaptation to stress depends on the modulation of gene expression. Regulation of mRNA
41 stability and degradation in stress granules (SGs), - cytoplasmic membraneless organelles
42 composed of messenger ribonucleoprotein (mRNP) complexes, - plays an important role in
43 fine-tuning of gene expression. In addition, SG formation can modulate stress signaling
44 pathways by protein sequestration. Molecular composition, structure, and function of SGs in
45 plants remain obscure. Recently, we established Tudor Staphylococcal Nuclease (TSN or
46 Tudor-SN; also known as SND1) as integral component of SGs in *Arabidopsis thaliana*. Here,
47 we combined purification of TSN interactome with cell biology, reverse genetics and
48 bioinformatics to study composition and function of SGs in plants. We found that under both
49 normal (in the absence of stress) and stress conditions TSN interactome is enriched in the
50 homologues of known mammalian and yeast SG proteins, in addition to novel or plant-specific
51 SG components. We estimate that upon stress perception, approximately half of TSN
52 interactors are recruited to SGs *de novo*, in a stress-dependent manner, while another half
53 represent a dense protein-protein interaction network pre-formed before onset of stress. Almost
54 all TSN-interacting proteins are moderately or highly disordered and approximately 20% of
55 them are predisposed for liquid-liquid phase separation (LLPS). This suggests that plant SGs,
56 similarly to mammalian and yeast counterparts, are multicomponent viscous liquid droplets.
57 Finally, we have discovered that evolutionary conserved SNF1-related protein kinase 1
58 (SnRK1) interacts with TSN in heat-induced SGs and that SnRK1 activation critically depends
59 on the presence of TSN and formation of SGs. Altogether, our results establish TSN as a
60 docking platform for SG-associated proteins and important stress signal mediator in plants.

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63 **Keywords:** intrinsically disordered region (IDR), liquid-liquid phase separation (LLPS),
64 messenger ribonucleoprotein (mRNP) complex, RNA-binding protein (RBP), SG proteome,
65 stress granule (SG), SNF1-related protein kinase 1 (SnRK1), Tudor Staphylococcal Nuclease
66 (TSN).

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68

69 INTRODUCTION

70 Upon stress perception, eukaryotic cells compartmentalize specific mRNA molecules stalled in
71 translation initiation in two types of evolutionarily conserved membraneless organelles (MLOs)
72 called stress granules (SGs) and processing bodies (PBs) (Thomas et al., 2011; Protter and
73 Parker, 2016). In these organelles, mRNA molecules are stored, degraded or kept silent to
74 prevent energy expenditure on producing useless, surplus or even harmful proteins under stress
75 conditions.

76
77 Recent research in budding yeast *Saccharomyces cerevisiae* and animal models established the
78 molecular composition of SGs and PBs. SGs typically contain poly(A)+ mRNA, 40S ribosomal
79 subunits, various translation initiation factors (eIF), poly(A)-binding protein (PABP) and a
80 variety of RNA-binding proteins (RBPs) and non-RNA-binding proteins (Buchan and Parker,
81 2009). PBs contain proteins belonging to the mRNA decay machinery, including subunits of
82 decapping and exosome complexes (DCP and XRN proteins, respectively), deadenylases and
83 many RBPs (Franks and Lykke-Andersen, 2008). Although there is a dynamic flux of proteins
84 and mRNA molecules between SGs and PBs, these MLOs have different functions. SGs play a
85 major role in translational repression by sequestering, stabilizing and storing mRNA molecules,
86 as well as by indirectly modulating signaling pathways, whereas PBs are to a large extent
87 involved in mRNA decay (Protter and Parker, 2016; Mahboubi and Stochaj, 2017).

88
89 Apart from components of SGs and PBs, proteomic and genetic screens in yeast and animal
90 models have also identified proteins modulating their assembly, which is a highly coordinated
91 process driven by numerous proteins (Ohn et al., 2008; Buchan et al., 2011; Martinez et al.,
92 2013; Jain et al., 2016). A recent model for the formation of mammalian and yeast SGs suggest
93 that they assemble in a two-step process, first involving the formation of a dense stable SG core
94 followed by accumulation of proteins containing intrinsically disordered regions (IDRs) into a
95 peripheral shell, the process involving liquid-liquid phase separation (LLPS) (Jain et al., 2016;
96 Markmiller et al., 2018).

97
98 In plants, molecular composition and function of SGs, as well as pathways of their assembly
99 and cross-talk with other signaling pathways remain largely unknown. Previous studies in
100 *Arabidopsis thaliana* (*Arabidopsis*) revealed formation of SGs under heat, hypoxia and salt
101 stress (Sorenson and Bailey-Serres, 2014; Yan et al., 2014; Gutierrez-Beltran et al., 2015). To
102 date, few conserved components of plants SGs have been identified, most of which are

103 homologues of yeast and/or mammalian SG-associated proteins. These include T-cell-restricted
104 intracellular antigen-1 (TIA-1) homologues Rbp47 and oligouridylate-binding protein 1
105 (Ubp1), endoribonuclease G3BP homologue nuclear transport factor 2 (NTF2-RPM) and
106 tandem CCCH zinc finger (TZF) family of RBPs (Bogamuwa and Jang, 2013; Sorenson and
107 Bailey-Serres, 2014; Gutierrez-Beltran et al., 2015; Krapp et al., 2017; Kosmacz et al., 2018).

108
109 Interestingly, Tudor Staphylococcal Nuclease (TSN) has been established as a novel component
110 of SGs in such distant lineages as protozoa, animals and plants (Zhu et al., 2013; Yan et al.,
111 2014; Gao et al., 2015; Gutierrez-Beltran et al., 2015; Cazares-Apatiga et al., 2017). The
112 domain composition of TSN includes tandem repeat of four Staphylococcal Nuclease (SN)
113 domains at its N-terminus followed by a Tudor and C-terminal, partial SN domain (Abe et al.,
114 2003; Gutierrez-Beltran et al., 2016). To date, TSN is known to be critically involved in the
115 regulation of virtually all pathways of gene expression, ranging from transcription to RNA
116 silencing (Gutierrez-Beltran et al., 2016). The interaction of TSN with proteins forming the
117 core of SGs, such as Pabp1, eIF4E, eIF5A, TIAR (TIA1-related protein) and TIA1 (T-cell-
118 restricted intracellular antigen) in different organisms indicate that TSN is an evolutionary
119 conserved component of SGs (Weissbach and Scadden, 2012; Zhu et al., 2013; Gao et al.,
120 2014).

121
122 In yeast and mammals, the universal molecular components of SGs co-exist together with other
123 cell type- and stress stimulus-specific components, suggesting that SGs might play additional,
124 yet unexplored, roles during stress. Thus, SG assembly in both yeast and human cells affects
125 target of rapamycin (TOR) signaling by sequestering both complex 1 TOR and downstream
126 kinases to alter signaling during stress (Takahara and Maeda, 2012; Wippich et al., 2013). On
127 the other hand, sequestration of the pleiotropic adaptor protein Receptor For Activated C Kinase
128 1 (RACK1) in SGs inhibits the stress-induced activation of the c-Jun N-terminal kinases (JNK)
129 cascade that triggers apoptotic death (Arimoto et al., 2008). In another scenario, sequestration
130 of the coiled-coil containing protein kinase 1 (ROCK1) into SGs promotes cell survival by
131 abolishing JNK-mediated cell death (Tsai and Wei, 2010). In summary, SG formation can
132 control signaling pathways by protein sequestration during stress conditions, but whether such
133 mode of regulation exists in plants remains elusive.

134
135 In the present study, we isolated TSN-interacting proteins from *Arabidopsis* plants subjected to
136 different types of stresses, and further combined microscopy, reverse genetics and

137 bioinformatics to advance our understanding of the regulation and molecular function of SGs
138 in plants. We show that SG proteins form a dense interaction network already under normal (no
139 stress) conditions that is poised to enable rapid SG assembly in response to stress. We found
140 that TSN functions as a platform for docking homologues of key components of yeast and
141 mammalian SGs, as well as novel or plant-specific components. Ubiquitous occurrence of
142 intrinsically-disordered proteins (IDPs) among TSN interactors supports a notion of LLPS
143 being a key process underlying SG assembly in plants. Finally, we have discovered that TSN
144 and formation of SGs confer heat-induced activation of the major energy sensor SNF1-related
145 protein kinase 1 (SnRK1).

146

147 **RESULTS**

148

149 ***Generation and characterization of Arabidopsis TAPa-expressing lines***

150 As a first step to investigate the role of TSN in SG formation, we have used TSN2 *Arabidopsis*
151 isoform as a bait for alternative tandem affinity purification (TAPa) (Rubio et al., 2005). Thus,
152 TSN2 and green fluorescent protein (GFP; negative control) were tagged at their C-termini with
153 TAPa epitope containing two copies of the immunoglobulin-binding domain of protein A from
154 *Staphylococcus aureus*, a human rhinovirus 3 protease cleavage site, a 6-histidine repeat and
155 9-myc epitopes (Figure 1A). Resulting TSN2-TAPa and GFP-TAPa vectors were introduced
156 into *Arabidopsis* Columbia (Col) background. Two lines per construct showing good
157 expression levels were selected for further studies (Supplemental Figure S1A).

158

159 To verify whether the presence of TAPa epitope could affect intracellular localization of TSN
160 protein, we performed immunostaining of control and heat-stressed root cells using α -Myc. The
161 analysis revealed that similar to native TSN (Yan et al., 2014; Gutierrez-Beltran et al., 2015),
162 TSN2-TAPa had diffused cytoplasmic localization under no stress (NS) conditions but
163 redistributed to punctate foci following heat stress (HS) (Figure 1B). In contrast, GFP-TAPa
164 remained cytoplasmic regardless of conditions (Figure 1B). These data suggested that C-
165 terminally TAPa-tagged TSN seemed to preserve key functional features of its native
166 counterpart when expressed in *Arabidopsis*, thus representing physiologically-relevant bait for
167 the isolation of TSN-interacting proteins.

168

169

170 ***Identification of TSN2-interacting proteins***

171 To examine the feasibility and efficiency of purifying TSN2-TAPa and GFP-TAPa proteins
172 from the corresponding transgenic plants, we performed a small-scale TAPa purification by
173 following the TAPa purification procedure (Figure S2). As shown in Figure S3A, the
174 immunoblot analysis with α -Myc confirmed that both TAPa-tagged proteins could be properly
175 purified. Since TSN2 was originally identified as a robust marker of SGs induced by HS in
176 *Arabidopsis* plants (Gutierrez-Beltran et al., 2015), we anticipated to initially compare the
177 TSN2 interactomes under HS and NS conditions. This comparison enabled classification of
178 various TSN interactors into one of three classes (Figure 1C): (i) stress-independent interactors,
179 which associate with TSN independently of HS; (ii) stress-dependent interactors, which
180 associate with TSN only under HS; and (iii) stress-sensitive interactors, whose association with
181 TSN is lost during HS.

182 The mass spectrometry analysis yielded 1,091 and 4,490 hits under NS conditions and 1,493
183 and 1,573 hits under HS conditions in GFP and TSN2 isolations, respectively (Figure S4). In
184 order to identify specific interactors of TSN2, we filtered the results using a two-step procedure.
185 First, we removed GFP-interacting proteins as well as proteins that are not present in at least
186 two biological replicates (non-specific interactions in Figure S4). The only exception was made
187 for some of the well-known plant SG components, such as PAB4, Rbp47 or RHM, which were
188 kept in the list regardless of their appearance in the GFP sample. Thereafter, proteins were
189 filtered based on subcellular localization according to The *Arabidopsis* Subcellular Database
190 (Tanz et al., 2013), excluding proteins found in the chloroplasts or mitochondria (subcellular
191 localization in Figure S4). As a result, we obtained the lists of 315 and 176 proteins representing
192 physiologically relevant interactomes of TSN2 under NS and HS settings, respectively (Figure
193 S4 and Supplemental Table 1)

194
195 A comparative analysis of KEGG orthologs [KO; (Nakaya et al., 2013)] revealed that ~ 28%
196 proteins from both TSN2_NS and TSN2_HS pools are known components of human or yeast
197 SGs (Figure 2A and Supplemental Figure S5A) (Jain et al., 2016). Similarly, subsets of 20 or
198 21 proteins from either pool of TSN2 interactors were shared with the recently reported
199 *Arabidopsis* Rbpb47b interactome (Figure S5B) (Kosmacz et al., 2019). An *in silico* analysis
200 found a significant degree of similarity among TSN2_NS pool, TSN2_HS pool and both yeast
201 and human SG proteomes in regards to functional distribution of composite proteins. Thus, both
202 TSN2_NS and TSN2_HS pools are enriched in RNA-binding proteins (RBPs), proteins with

203 predicted prion-like domains (PrLDs) and proteins with ATPase activity (Figure 2B,
204 Supplemental Table 2). We could not find known PB components such as DCP or XRN family
205 proteins among TSN2 interactors, indicating that TSN specifically binds SG components
206 (Maldonado-Bonilla, 2014; Youn et al., 2018). Collectively these data demonstrate the
207 robustness of our approach, in which TSN2 was chosen as bait for identification of plant SG-
208 associated components.

209

210 ***TSN interactome reveals a pre-existing network of SG protein-protein interactions***

211 Although SGs are only microscopically visible under stress conditions (Jain et al., 2016),
212 analysis of the TSN2_NS and the TSN_HS protein pools revealed that both pools are
213 significantly enriched in known mammalian and/or yeast SG components (Group 1, Figure 2C),
214 and also in proteins found in the previously characterized *Arabidopsis* Rbp47b proteome
215 (Kosmacz et al., 2019), such as PAB8, SKD1, IF4A3 or RHM2 (Group 2, Figure 2C).
216 Furthermore, 74% (235/315) protein hits of the TSN2_NS pool were absent from the TSN2_HS
217 pool, thus representing HS-sensitive part of the TSN2 interactome. Accordingly, the remaining,
218 smaller part of the TSN2_NS pool (26%, 81/315) was shared with the TSN2_HS pool,
219 corresponding to stress-independent TSN2 interactors (Figure 2C). The latter class of proteins
220 included TSN1, UBP1c, Rbp47, PAB4, VCS or TCTP, among others. Lastly, 54% (96/177)
221 protein hits from the TSN2_HS pool, including HSP70 and individual subunits of both EIF4
222 translation initiation factors and EF1elongation factors were absent from the TSN2_NS pool,
223 and therefore represented HS-dependent TSN2 interactors (Figure 2C).

224

225 To expand on these findings, we also retrieved publicly available direct protein-protein
226 interaction (PPI) data for all proteins found in our proteomic studies. Both TSN2_NS and
227 TSN2_HS pools formed a dense network of protein-protein interactions (Figure 2D), containing
228 315 and 176 nodes and 885 and 469 edges respectively. In this context, the average number of
229 interactions per protein for these two pools was 5.4 ($p = 5.63 \times 10^{-11}$) and 4.1 ($p = 2.17 \times 10^{-10}$),
230 respectively. Together with our previous observations that the core SG proteins such as Rbp47,
231 Ubp1 or PAB4 interact with TSN in *Arabidopsis* cells in the absence of stress, these results
232 point to a pre-existing steady-state network of protein interactions as a primordial mechanism
233 during SG formation, where TSN could act as an assembly platform.

234

235

236

237 ***TSN interactome is enriched in IDPs***

238

239 Studies in mammalian and yeast cells have suggested that SGs are multicomponent viscous
240 liquid droplets formed in the cytoplasm by LLPS (Kroschwald et al., 2015; Protter and Parker,
241 2016). Although the molecular details underlying LLPS in cells are largely obscure, recent
242 evidence suggests that IDRs mediate this process (Molliex et al., 2015; Wheeler et al., 2016).
243 In this context, we first evaluated the occurrence of proteins with IDRs in both TSN2_NS and
244 TSN2_HS interactomes using two predictor algorithms: PONDR-FIT and PONDR-VSL2
245 (Peng et al., 2005; Xue et al., 2010). The analysis revealed significant enrichment of both
246 interactomes in IDR-containing proteins (Figure 3A). Based on their intrinsic disorder (ID)
247 content, proteins were classified as highly ordered (disorder score < 0.25), moderately
248 disordered (disorder score between 0.25 and 0.5) and highly disordered (disorder score > 0.5).
249 According to PONDR-VSL2, while as much as 93% of the entire *Arabidopsis* proteome is
250 represented by moderately and highly disordered proteins, this frequency was increased further
251 in both TSN2_NS and TSN2_HS interactomes, reaching 99.4% and 100%, respectively (Figure
252 3B and Supplemental Figure S6).

253

254 Next, we evaluated the correlation between ID content and predisposition to undergo LLPS. As
255 shown in Figure 3C ~ 20% of proteins from both TSN2_NS (56/315) and TSN2_HS (36/177)
256 bear propensity for LLPS (score > 0.5), and most of them are moderately (disorder score
257 between 0.1 and 0.3) or highly (disorder score > 0.3) disordered. Taken together, the ubiquitous
258 occurrence of IDRs among the TSN2-interacting proteins reinforces the view that TSN is a
259 scaffolding factor seeding SG protein complexes.

260

261 In mammalian cells, highly disordered proteins such as TIA-1 are required to promote SG
262 formation via LLPS (Protter and Parker, 2016; Rayman et al., 2018). Considering this fact as
263 well as that TSN was shown to modulate the integrity of SGs in *Arabidopsis* (Gutierrez-Beltran
264 et al., 2015), we evaluated the per-residue ID propensities of TSN2 using a set of six commonly
265 used predictors, including PONDR-VLXT, PONDR-VL3, PONDR-VSL2, IUpred_short,
266 IUpred_long and PONDR-FIT (Meng et al., 2015). Figure 3D shows that TSN2 is expected to
267 have several (11 if averaged for six predictors) disordered regions (score above 0.5). Thus, the
268 SN domains of TNS2 are predicted to be highly disordered, whereas the tudor region is
269 predicted to be one of the most ordered parts of this protein. This observation was confirmed
270 using D²P² database providing information about the predicted disorder and selected disorder-

271 related functions (Supplemental Figure S7A)(Oates et al., 2013). Notably, similar results were
272 obtained for TSN1 protein isoform that is considered to be functionally redundant with TSN2
273 (Supplemental Figure S7) (dit Frey et al., 2010). Taken together, above results prompt us to
274 believe that TSN proteins may recruit SG components via IDR regions, promoting in this way
275 rapid coalescence of microscopically visible SGs upon stress exposure.

276

277 ***TSN-interacting proteins co-localize with TSN2 in cytoplasmic foci***

278 To ascertain the SG localization of TSN2-interacting proteins identified by mass spectroscopy,
279 we shortlisted the 16 most interesting proteins from the HS-independent class of interactors
280 (Figure 2C and 4A). The short list included homologues of key components of yeast and animal
281 SGs (IF4E5, PAB4 and a 40S ribosomal subunit) and hypothetical plant-specific SG
282 components with a key role in fundamental eukaryotic pathways (e.g. SnRK1 proteins, RH12,
283 SKP1, MC1 and TCTP). First, we performed a co-localization study to investigate whether the
284 shortlisted TSN-interacting proteins were translocated to TSN2 foci under stress. To this end,
285 protoplasts were isolated from *Nicotiana benthamiana* (*N. benthamiana*) leaves co-transformed
286 with RFP-TSN2 and individual GFP-TSN-interacting proteins. Co-transformation of the
287 cytoplasmic protein GFP-ADH2 and the SG marker GFP-Ubp1c with RFP-TSN2 were used as
288 a negative and positive control, respectively (Figure 4B, C). To quantify colocalization results,
289 we calculated the linear Pearson (r_p) and the nonlinear Spearman's rank (r_s) correlation
290 coefficient (PSC) for the pixels representing the fluorescence signals in both channels (Figure
291 4C). The levels of co-localization can range from +1 for positive correlation to -1 for negative
292 correlation (French et al., 2008). As shown in Figures 4B, C and S8, all shortlisted proteins co-
293 localized with TSN2 in punctate foci under HS.

294

295 Next, to elucidate whether these proteins interact with TSN2 in the heat-induced SGs, we
296 performed bimolecular fluorescence complementation (BiFC) analysis with 10 of 16 shortlisted
297 proteins. This analysis confirmed that all proteins indeed interacted with TSN2 in cytoplasmic
298 foci under stress (Figure 4D). Notably, we observed that, in line with our proteomics data, TSN2
299 interacted with its partners also under normal (NS) conditions (Supplemental Figure S9). Taken
300 together, these findings suggest that a subset of proteins that respond to stress by re-localization
301 to punctate foci interact with TSN and are thus new candidates for SG-associated components
302 in plants.

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304

305 ***N-terminally situated SN domains of TSN participate in the interaction with SG proteins***

306 To investigate whether the interaction of TSN with SG proteins presents any domain
307 preference, we performed a BiFC assay with full-length TSN2 or either SN region (tandem
308 repeat of four N-terminally located SN domains) or Tudor (Tudor and the fifth SN domains)
309 region (Figure 4E) fused to cYFP and four different SG-associated TSN-interacting proteins
310 fused to nYFP in heat-stressed *N. benthamiana* leaves (Figure 4F). The results have revealed
311 reconstitution of fluorescent signal in the experiments with all four TSN interactors in case of
312 both full-length TSN2 and SN region, whereas none of the TSN interactors could form a
313 complex with Tudor region (Figure 4F). Furthermore, expression of either full-length TSN2 or
314 SN region yielded identical, punctate BiFC localization pattern. We conclude that tandem
315 repeat of four SN domains confers TSN ability to recruit partner proteins to SGs.

316

317 ***Arabidopsis SG components are common targets of TSN1 and TSN2 isoforms***

318 *Arabidopsis* TSN1 and TSN2 proteins have been previously considered as functionally
319 redundant (dit Frey et al., 2010; Gutierrez-Beltran et al., 2015). To investigate whether this
320 redundancy is conserved at SG level, we isolated TSN1 interactome from unstressed plants
321 using the same TAPa procedure as described above for TSN2 (Supplemental Figure S1B, S2,
322 S3B). As a result, we obtained TSN1_NS pool comprised of 270 protein hits (Supplemental
323 Figure S4 and Supplemental Table 1). Out of these, 108 (40%) were TSN1-specific, whereas
324 the remaining, larger fraction (164 proteins, 60%) represented common interactors of TSN1
325 and TSN2, reflecting their functional redundancy (Figure 5A). Notably, the pool of common
326 interactors of TSN1 and TSN2 was enriched in SG proteins, including core, evolutionarily
327 conserved components such as PAB4, 40S ribosomal subunits, DEAD-box helicases or CCT
328 proteins (group 1, Figure 5A). In addition to known SG homologs in either human or yeast, the
329 TSN1/2 pool contains proteins found in the previously characterized *Arabidopsis* Rbp47b
330 proteome (group 2), as well as novel plant SG components verified through either co-
331 localization or BiFC with TSN2 or both methods (group 3, Figure 4A, C, D; Supplementary
332 Figure S8).

333

334 To corroborate proteomics results, we have chosen DEAD-box ATP-dependent RNA helicase
335 12 (RH12), as a common interactor of TSN1 and TSN2. First, we confirmed molecular
336 interaction between two isoforms of TSN and RH12 by coimmunoprecipitation (Co-IP) in cell
337 extracts from agro-infiltrated *N. benthamiana* leaves. As shown in Figure 5B, RH12
338 coimmunoprecipitated with both TSN1 and TSN2 but did not with GFP, used as a negative

339 control in this experiment. Second, we produced *Arabidopsis* lines stably expressing GFP-
340 RH12 under native promoter and observed relocalization of the fusion protein to cytoplasmic
341 foci under HS conditions in root tip cells (Figure 5C). Taken together, these data indicate that
342 TSN1 and TSN2 are likely redundant in providing a scaffold platform for the recruitment of a
343 wide range of SG components in *Arabidopsis* plants.

344

345 ***Isolation of salt stress-induced TSN2 interactome and candidate core SG components***

346 Previously it was shown that TSN2 is re-localized to SGs under salt stress (Yan et al., 2014).
347 To investigate how TSN2 interactome is affected by salt stress, we isolated TSN2_NaCl
348 interactome from salt-stressed *Arabidopsis* plants using our standard TAPa purification
349 procedure. The resulting TSN2_NaCl pool included significantly lower number of protein hits
350 (44 hits), as compared to both TSN2_NS and TSN2_HS pools hits (Figure 6A; Supplemental
351 Figure S4 and Supplemental Table 1). Fifty two percent (23/44) of the protein hits were
352 classified as NaCl stress-independent TSN2 interactors, as they also appeared in the TSN2-NS
353 dataset, including many well-characterized SG proteins (e.g., Rbp47, UBP1, PAB4, several
354 helicases and 40S ribosomal subunits). Interestingly, no known SG component were found
355 among NaCl stress-dependent TSN2 interactors (Supplemental Table 1), suggesting a new role
356 for TSN2 under salt stress that could be explored in future studies.

357

358 A broader comparative analysis including all three pools of TSN2 interactors, i.e. TSN2_NS,
359 TSN2_HS and TSN2_NaCl yielded eleven proteins present in all three pools and presumably
360 representing core SG components constitutively bound to the TSN platform (Figure 6B). To
361 validate this assumption, we performed Co-IP using protein extracts prepared from 7-day-old
362 *Arabidopsis* seedlings expressing GFP-Rbp47 (a putative core SG protein fused to GFP) and
363 exposed to heat or salt stress (Rayman et al., 2018). As shown in Figure 6C, TSN co-
364 immunoprecipitated with GFP-Rbp47 under both types of stresses, as well as in the absence of
365 stress. To further corroborate our result by *in planta* observations, we produced *Arabidopsis*
366 lines stably expressing GFP-fused variants of Rbp47 and UBP1, two putative core SG proteins,
367 and TCTP and SnRK1.2, two HS-dependent TSN2 interactors. Analysis of root tip cells
368 revealed that while Rbp47b and UBP1 were localized to both HS- and NaCl-induced SG puncta,
369 TCTP and SnRK1.2 exhibited punctate localization only under HS (Figure 6D).

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373 ***TSN and SGs confer heat-induced activation of SnRK1***

374 We have found that SnRK1.1 and SnRK1.2, - two *Arabidopsis* homologues of the evolutionary
375 conserved SNF1-related protein kinase 1, - are novel TSN-interacting proteins re-localized to
376 SGs exclusively during HS (Figure 4 and Figure 6D). To dissect the functional relevance of
377 TSN binding and SG localization of SnRK1.1 and SnRK1.2, we investigated whether HS and
378 the presence of TSN could affect their kinase activity. To begin with, we corroborated the
379 interaction with TSN2 using two different approaches. First, we performed co-
380 immunoprecipitation of native TSN and GFP-SnRK1.2 in protein extracts prepared from heat-
381 stressed *Arabidopsis* plants expressing GFP-SnRK1.2. We found that native TSN co-
382 immunoprecipitated with GFP-SnRK1.2 but not with GFP, which was used as a negative
383 control (Figure 7A). Second, a Förster resonance energy transfer (FRET) assay demonstrated
384 that TSN2 directly interacts with SnRK1.2 in *N. benthamiana* leaves under HS (Figure 7B).
385 Taken together, these findings confirm the *in vivo* TSN-SnRK1 interaction.

386
387 To determine whether SnRK1 activity is regulated *in vivo* by HS, we subjected 10-day-old
388 Columbia (Col) *Arabidopsis* seedlings to 39°C for 0, 20, 40 and 60 min, and performed
389 immunoblotting using α -phospho-AMPK Thr175 (α -pT175), which recognizes both SnRK1.1
390 (upper band 61.2 kDa) and SnRK1.2 (lower band 58.7 kDa) (Rodrigues et al., 2013; Nukarinen
391 et al., 2016). As a control test, we confirmed the α -pT175 sensitivity using ABA treatment
392 known to induce SnRK1 T175 phosphorylation (Figure S10) (Jossier et al., 2009). Time course
393 analysis of the level of SnRK1 T175 phosphorylation under HS demonstrated that the two
394 SnRK1 isoforms are rapidly activated by stress (Figure 7C). To exclude the possible bias due
395 to fluctuations in the protein expression level, we conducted immunoblotting assays using α -
396 SnRK1.1 and SnRK1.2, and found that the protein levels of both kinases were not affected by
397 the treatment. To further correlate the SnRK1 activity with the formation of SGs, the seedlings
398 were treated with cycloheximide (CHX), a drug blocking SG assembly in mammalian and plant
399 cells (Gutierrez-Beltran et al., 2015; Wolozin and Apicco, 2015). As shown in Figure 7D, CHX
400 treatment abrogated heat-induced phosphorylation of SnRK1 T175, suggesting that activation
401 of SnRK1 isoforms is linked to the formation of heat-induced SGs.

402
403 To test whether TSN is involved in the regulation of the SnRK1 kinase activity, *tsn1tsn2* double
404 mutant seedlings were exposure to 39°C for 0, 20, 40 and 60 min, and protein extracts were
405 analyzed by immunoblotting with α -pT175. As in case of CHX treatment, TSN deficiency
406 prevented heat-induced phosphorylation of SnRK1 T175 (Figure 7E). Accordingly,

407 complementation of the *tsn1tsn2* double mutant with TSN2 under the native promoter resulted
408 in full rescue of the heat-induced phosphorylation of SnRK1 T175 (Figure 7F), confirming that
409 TSN is required for the activation of SnRK1 during HS.

410

411 **DISCUSSION**

412

413 One of the earliest, evolutionary conserved events occurring upon stress perception and
414 providing defence mechanism to promote cell survival is the assembly of SGs in the cytoplasm
415 of eukaryotic cells (Thomas et al., 2011; Mahboubi and Stochaj, 2017). Molecular composition
416 and regulation of SGs is a rapidly growing area, but most of the works done so far utilized
417 animal or yeast models.

418

419 In a recent study, we found that TSN is stably associated with SGs in *Arabidopsis* (Gutierrez-
420 Beltran et al., 2015). According to current hypothesis suggesting that the SG cores are relatively
421 stable, while the SG shells are highly dynamic (Jain et al., 2016), we hypothesize that TSN is a
422 SG core protein in plants. In the present study, we found that the *Arabidopsis* TSN proteins
423 interact with numerous SG components and that most of these interactions take place under no
424 stress condition (Figure 2). This finding, together with the fact that N-terminally situated SN
425 domains are essential for these interaction, as well as SG-specific localization of TSN (Zhu et
426 al., 2013; Gutierrez-Beltran et al., 2015), make it reasonable to propose the potential role of the
427 SN domains as a docking platform maintaining a pre-existing state of SGs in plant cells (Figure
428 8).

429

430 There are several lines of evidence suggesting that assembly of mammalian and yeast SGs
431 might be a highly regulated process controlled, at least in part, by ATP-dependent remodeling
432 complexes (Protter and Parker, 2016). First, numerous energy-driven chaperones have been
433 found in SG proteomes. Second, ATP is required for the formation of SGs (Jain et al., 2016).
434 Therefore, ATP-dependent events mediated by ATPases, such as movement of mRNPs to sites
435 of SG formation by motor proteins or remodeling of mRNPs to load required components could
436 be imperative for promoting SG assembly. In this context, the interaction of chaperonin-
437 containing T complex (CCT complex) with SG components has been suggested to be crucial
438 for the proper assembly of SGs in yeast (Jain et al., 2016). Similarly, a mutation of DEAD-box
439 helicase 1 (Ded1) that blocks its ATPase activity leads to retention of mRNAs in SGs and
440 accumulation of these foci in the cytoplasm of yeast cells (Hilliker et al., 2011). In plants, the

441 homologues of the yeast DEAD-box RNA helicase DHH1 is required for proper formation of
442 stress granules (Chantarachot. et al., 2019). Considering that an enrichment of the TSN
443 interactome in ATP-dependent remodeling complexes, including CCT proteins and DEAD-box
444 RNA/DNA helicases occurs exclusively in the absence of stress stimulus (Figure 2C), we
445 hypothesize that interaction between these proteins and TSN is necessary for the early steps of
446 SG assembly in plants (Figure 8). Once the stress stimulus is perceived, the ATP-dependent
447 remodeling complexes might detach from the TSN platform and aid in SG shell assembly.

448
449 The composition of SG proteome in animal and yeast cells is a highly variable characteristics
450 influenced by a type of stress or cell type (Markmiller et al., 2018). However, certain proteins,
451 e.g. G3BP1 and PAB, are constant SG constituents (Mahboubi and Stochaj, 2017). We
452 estimated that up to 50% of TSN-interacting proteins may be recruited to *Arabidopsis* SGs in
453 stress type-specific manner. In addition to a large resource of nearly 400 previously unknown
454 plant candidate SG proteins for further validation, our study provides also a subset of proteins
455 constantly interacting with TSN, regardless of stress or a type of stress. These proteins,
456 including UBP1, PAB4, Rbp47 or RH12 can therefore be considered as core constituents of
457 plant SGs (Figure 6B).

458
459 In non-plant species, one of the most enriched categories of molecular components of SGs are
460 RBPs, regulating RNA transport, silencing, translation and degradation (Wolozin and Apicco,
461 2015). Likewise, RBPs accounted for 53% and 54% of TSN2_HS and TSN2_NS interactomes
462 (Figure 2B), respectively, providing a further mechanistic explanation for the previously
463 established role of TSN in mRNA stabilization and degradation (Gutierrez-Beltran et al., 2015).
464 Yet, we found a high occurrence of ribosomal proteins, including components of 40S subunits,
465 consistently with several studies showing a close association between ribosomes and SGs
466 (Yang et al., 2014; Cary et al., 2015). Curiously, we have not found any PB-specific proteins,
467 such as DCP or XRN, among *Arabidopsis* TSN interactors. In contrast, proteins such as VCS,
468 PATL1 and several helicases, which have been localized to both SG and PB cytoplasmic foci
469 (Youn et al., 2018) were identified in the TSN interactome, suggesting that similarly to yeast
470 and animal models, SGs and PBs in plants share a part of their components.

471
472 A current, predominating model for SG assembly rests on LLPS driven by dynamic and
473 promiscuous interactions among IDRs (Molliex et al., 2015; Rayman et al., 2018). In this
474 context, the overexpression of the prion-like domain (a type of IDR) of the mammalian SG core

475 protein TIA-1 was sufficient to promote the formation of SGs (Kedersha et al., 1999; Gilks et
476 al., 2004). In *Arabidopsis* plants, the prion-like domains of the TIA-1 homologues UBP1 and
477 RBP47 were required for protein targeting to SGs (Weber et al., 2008). Our data further
478 demonstrate that almost all *Arabidopsis* TSN-interacting proteins are disordered (Figure 3B),
479 and ~ 20% of them are predisposed for LLPS (Figure 3C). Lastly, TSN protein itself is highly
480 disordered, with the most IDRs located within its five SN domains (Figure 3D), four of which,
481 situated at the N-terminus were shown to confer TSN interaction with partner proteins, SG
482 localization and cytoprotective function in both mammalian and plant cells (Figure 4F; (Gao et
483 al., 2015; Gutierrez-Beltran et al., 2015). Taken together, these results have two important
484 implications. First, the function of IDRs in SG condensation is conserved in plants. Second, the
485 IDRs of TSN-dependent protein complexes may underpin SG functions.

486
487 It is well known that numerous stress- and nutrient-signaling pathways converge on SGs
488 (Kedersha et al., 2013; Mahboubi and Stochaj, 2017). Our study has established two
489 homologues of the evolutionary conserved signaling protein SnRK1 (SnRK1.1 and SnRK1.2;
490 also known as KIN11 and KIN10, respectively) as TSN interactors. SnRK1 (AMPK in
491 mammals and Snf1 in yeast) has been extensively studied as one of the key regulators of the
492 target of rapamycin (TOR) (Shaw, 2009). In plants, SnRK1 and TOR proteins play central and
493 antagonistic roles as integrators of transcription networks in stress and energy signaling (Baena-
494 Gonzalez et al., 2007). Thus, while SnRK1 signaling is activated during stress and energy
495 limitation, TOR promotes growth and biosynthetic processes in response to energy availability
496 (Baena-Gonzalez and Hanson, 2017; Carroll and Dunlop, 2017). Although it has been
497 demonstrated that the mammalian orthologue (AMPK) is a *bona fide* SG component involved
498 in the regulation of SG biogenesis (Mahboubi et al., 2015), there is no evidence connecting
499 SnRK1 activation and SGs. Here we demonstrate that formation of SGs and the presence of
500 TSN are both necessary for SnRK1 activation in response to HS (Figure 7). It has been shown
501 that mammalian mTOR is translocated to SGs under stress, leading to its inactivation (Heberle
502 et al., 2015). While there is no evidence so far that TOR is a component of SGs in plants, we
503 detected a TOR downstream effector RPS6 among TSN-interacting proteins (Figure 2C,
504 Supplemental Table 1). We thus speculate that SGs and their integral constituent protein TSN
505 might play a crucial role in the regulation of the SnRK1-TOR module; however further work is
506 required to decipher mechanistic details and physiological roles of this regulation.

507

508 Based on our current results, we propose a three-step working model for the TSN-dependent
509 biogenesis of plant SGs that encompasses formation of the pre-existing TSN-SG complex, as
510 the first, primordial step crucial for proper SG assembly (Figure 8). A few minutes after stress
511 exposure, a high-density protein-protein interaction network mediated by IDRs of stress-
512 independent TSN interactors induces LLPS, leading to the formation of a pre-assembled state.
513 Since TSN is a core component (Gutierrez-Beltran et al., 2015), one possibility is that formation
514 of stress granule core takes part first, followed by the assembly of a shell around this core in
515 which detachment of stress-sensitive TSN interactors and incorporation of stress-dependent
516 interactors accomplishing SG maturation process. ATP modulators present in the stress-
517 sensitive pool such as CCT or DEAD-box RNA/DNA helicases, could be critically required for
518 both transition steps.

519

520 **EXPERIMENTAL PROCEDURES**

521

522 ***Plant Material and Molecular Biology***

523 The T-DNA *tsn1tsn2* double mutant for *TSN1* and *TSN2*, in the Landsberg erecta (Ler) and
524 Columbia (Col) backgrounds, respectively, was isolated as shown previously (Gutierrez-
525 Beltran et al., 2015). The mutant was five times back-crossed with wild-type (WT) Col plants
526 to generate an isogenic pair. Finally, both *tsn1tsn2* mutant and WT plants were selected from
527 F5. All oligonucleotide primers used in this study are shown in Supplemental Table 3. *TSN1*
528 (no stop codon egb1/egb29; with stop codon egb1/egb28), *TSN2* (no stop codon egb4/egb6;
529 with stop codon egb4/egb5), and *GFP* (egb19/egb20) were amplified by PCR from pGWB6
530 and resulting cDNA sequences were introduced in pDONR/Zeo using Gateway technology
531 (Invitrogen). For expression of C-terminal TAPa fusion under the control of (2x) 35S promoter,
532 *TSN1*, *TSN2* and *GFP* cDNAs were introduced into the destination vector pC-TAPa (Rubio et
533 al., 2005). cDNA clones of TSN-interacting proteins in the Gateway compatible vector
534 pENTR223 were obtained from the ABRC stock center (Yamada et al., 2003). For expression
535 of N-terminal GFP and RFP fusions under the control of 35S promoter, cDNAs encoding *TSN2*
536 and TSN-interacting proteins were introduced into the destination vectors pMDC43 and
537 pGWB655, respectively (Curtis and Grossniklaus, 2003). For BiFC assay, cDNAs for *TSN2*,
538 TSN-interacting proteins, as well as SN and Tudor regions were cloned into pSITE-BiFC
539 destination vectors (Martin et al., 2009). For FRET experiments, cDNAs for *TSN2* and
540 SnRK1.2 were introduced into pGWB642 (YFP) and pGWB645 (CFP) destination vectors

541 (Nakamura et al., 2010). All plasmids and derived constructs were verified by sequencing using
542 the M13 forward and reverse primers.

543

544 ***Tandem Affinity Purification***

545 Fully expanded leaves from *Arabidopsis* Col transgenic plants expressing TSN-TAPa and GFP-
546 TAPa and grown in the greenhouse for 18 days in 18:6 light/dark conditions at 22 °C (NS),
547 39°C for 40 min (HS) and 200 mM NaCl₂ for 5 h (NaCl) were harvested (15 g, fresh weight)
548 and ground in liquid N₂ in 2 volumes of extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM
549 NaCl, 10% glycerol, 0.1% Nonidet P-40 and 1x protease inhibitor cocktail; Sigma-Aldrich) and
550 centrifuged for 12,000 g for 10 min at 4°C. Supernatants were collected and filtered through
551 two layers of Miracloth (Calbiochem). Plant extracts were incubated with 700 µL IgG beads
552 (Amersham Biosciences) for 4-5 h at 4°C with gentle rotation. After centrifugation at 250 g for
553 3 min at 4°C, the IgG beads were recovered and washed three times with 10 mL of washing
554 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, and 0.1% Nonidet P-40) and
555 once with 5 mL of cleavage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol,
556 Nonidet P-40, and 1 mM DTT). Elution from the IgG beads was performed by incubation with
557 15 µL (40 units) of PreScission protease (Amersham Biosciences) in 5 mL of cleavage buffer
558 at 4°C with gentle rotation. Supernatants were recovered after centrifugation at 250 g for 3 min
559 at 4°C and stored at 4°C. The IgG beads were washed with 5 mL of washing buffer, centrifuged
560 again, and the eluates pooled. Pooled eluates were transferred together with 1.2 mL of Ni-NTA
561 resin (Qiagen, Valencia, CA, USA) into a 15 mL Falcon tube and incubated for 2 h at 4°C with
562 gentle rotation. After centrifugation at 250 g for 3 min at 4°C, the Ni-NTA resin was washed
563 three times with 10 mL washing buffer. Finally, elution was performed using 4 mL of
564 imidazole-containing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1%
565 Nonidet P-40, 200 mM imidazole). All the steps in the purification procedure were carried out
566 at 4°C. For each large-scale TAPa purification, three TAPa plant samples (15 g, fresh weight
567 each) were processed in parallel as described above. Final eluates were pooled together,
568 proteins were precipitated using TCA/Acetone extraction and 100 µg of protein was digested
569 according to the FASP method (Wisniewski et al., 2009). Three and two biological replicates
570 were performed for isolating TSN interactomes from unstressed and stressed plants,
571 respectively.

572

573 ***Liquid chromatography and mass spectrometry analysis***

574 Peptides were analyzed using EASYnano-LC 1000 on a Q Exactive Plus Orbitrap mass
575 spectrometer (Thermo Scientific). Peptides were separated on a pre-column 75 μm x 2 cm,
576 nanoViper, C18, 3 μm , 100 \AA (Acclaim PepMap 100) and analytical column 50 μm x 15 cm,
577 nanoViper, C18, 2 μm , 100 \AA (Acclaim PepMap RSLC) at a flow rate of 200 nL/min. Water
578 and ACN, both containing 0.1% formic acid, were used as solvents A and B, respectively. The
579 gradient was started and kept at 0-35% B for 0-220 min, ramped to 35-45% B over 10 min, and
580 kept at 45-90% B for another 10 min. Operate the mass spectrometer in the data-dependent
581 mode (DDA), to automatically switch between full scan MS and MS/MS acquisition. Acquire
582 survey full scan MS spectra from 200 to 1800 m/z in the Orbitrap with a resolution of $R =$
583 70,000 at m/z 100. For data dependent analysis, the top 10 most abundant ions were analyzed
584 by MS/MS, while +1 ions were excluded, with a normalized collision energy of 32%. The raw
585 data were searched with the Sequest HT node of Proteome Discoverer 1.4. The Uniprot
586 database (*Arabidopsis* TAIR10), was utilized for the searches. All protein identification results
587 were filtered to include only high confidence peptides with peptide mass deviation 2 and a
588 minimum of 2 unique peptides per protein and score thresholds to attain an estimated false
589 discovery rate of ~1% using a reverse decoy database strategy (Chittum et al., 1998).

590 ***Plant and Protoplast Transformation***

591 *Arabidopsis* Columbia (Col) plants were transformed as described previously (Clough and
592 Bent, 1998) using *Agrobacterium tumefaciens* (*Agrobacterium*) strain GV3101. In Figure 5 and
593 6, plants from the T2 and T3 generations were used. Transgenic plants were confirmed by
594 genotyping. For transient expression in *N. benthamiana* mesophyll cells, *Agrobacterium* strain
595 GV3101 was transformed with the appropriate binary vectors by electroporation as described
596 previously (Gutierrez-Beltran et al., 2017). *Agrobacterium*-positive clones were grown in
597 Luria-Bertani until reaching $\text{OD}_{600} = 0.4$ and were pelleted after centrifugation at 3,000 g for 10
598 min. Cells were resuspended in MM (10 mM MES, pH 5.7, 10 mM MgCl_2 supplemented with
599 0.2 mM acetosyringone) until $\text{OD}_{600} = 0.4$, incubated at room temperature for 2 h, and infiltrated
600 in *N. benthamiana* leaves using a 1 mL hypodermic syringe. Leaves were analyzed after 48 h
601 using a Zeiss 780 confocal microscope with the 40x objective. The excitation/emission
602 wavelength was 480/508 nm for GFP, and 561/610 nm for RFP.

603 Protoplasts were isolated from leaves of 15- to 20-day-old *N. benthamiana*, transiently
604 expressing the corresponding fluorescent proteins, as described previously (Wu et al., 2009).
605 The cell walls were digested in enzymatic solution containing 1% (w/v) Cellulose R-10, 0.25%
606 (w/v) Macerozyme R-10, 20 mM MES-HOK pH 5.7, 400 mM Mannitol, 10 mM CaCl_2 , 20 mM

607 KCl, 0.1 % (w/v) Bovine serum albumin (BSA) for 60 min. Protoplasts were separated from
608 debris by centrifugation (100 g, 3 min, 4°C), washed two times with ice-cold W5 buffer (154
609 mM NaCl, 125 mM CaCl₂, 5 mM KCl and 2 mM MES-KOH pH 5.7) and resuspended in ice-
610 cold W5 buffer at a density of 2.5 x 10⁵ protoplasts mL⁻¹. The protoplast suspension was
611 incubated for 15 min on ice before heat stress.

612 ***Bimolecular Fluorescence Complementation (BiFC)***

613 For BiFC assays, *Agrobacterium* strains GV3101 carrying *cYFP-TSN2*, *cYFP-SN* or *cYFP-*
614 *Tudor* and the corresponding *nYFP-TSN-interacting proteins* were co-infiltrated into *N.*
615 *benthamiana* leaves (OD₆₀₀=0.3). Fluorescence images were obtained 48 h after infiltration
616 using a Leica TCS Sp2/DMRE confocal microscope, with excitation wavelength 514 nm.
617 Transient expression of proteins in *N. benthamiana* leaves via agroinfiltration was performed
618 as previously described (Gutierrez-Beltran et al., 2017).

619

620 ***Immunocytochemistry and Imaging***

621 Five-day-old *Arabidopsis* roots were fixed for 60 min at room temperature with 4% (w/v)
622 paraformaldehyde in 50 mM PIPES, pH 6.8, 5 mM EGTA, 2 mM MgCl₂, and 0.4% Triton X-
623 100. The fixative was washed away with phosphate buffered saline buffer supplemented with
624 Tween 20 (PBST) and cells were treated for 8 min at room temperature with a solution of 2%
625 (w/v) Driselase (Sigma-Aldrich) in 0.4 M mannitol, 5 mM EGTA, 15 mM MES pH 5.0, 1 mM
626 PMSF, 10 µg mL⁻¹ leupeptin, and 10 µg mL⁻¹ pepstatin A. Thereafter, roots were washed twice,
627 10 min each, in PBST and then in 1% (w/v) BSA in PBST for 30 min before overnight
628 incubation with a primary antibody (rabbit α-Myc diluted 1:500). The specimens were then
629 washed three times for 90 min in PBST and incubated overnight with goat anti-rabbit
630 fluorescein isothiocyanate (FITC) conjugated secondary antibody diluted 1:200. After washing
631 in PBST, the specimens were mounted in Vectashield mounting medium (Vector Laboratories).

632

633 ***Förster Resonance Energy Transfer (FRET)***

634 FRET was performed using Zeiss 780 laser scanning confocal microscope and a plan-
635 apochromat 20x/0.8 M27 objective. FRET acceptor photobleaching mode of Zeiss 780 ZEN
636 software was used, with the following parameters: acquisition of 10 pre-bleach images, one
637 bleach scan, and 80 post-bleach scans. Bleaching was performed using 488, 514 and 561 nm
638 laser lines at 100% transmittance and 40 iterations. Pre- and post-bleach scans were at minimum

639 possible laser power (0.8 % transmittance) for the 458 nm or 514 nm (4.7%) and 5% for 561
640 nm; 512 x 512 8-bit pixel format; pinhole of 181 μm and zoom factor of 2.0. Fluorescence
641 intensity was measured in the ROIs corresponding to the bleached region. One ROI was
642 measured outside the bleached region to serve as the background. The background values were
643 subtracted from the fluorescence recovery values, and the resulting values were normalized by
644 the first post-bleach time point. Three pre-bleach and three post-bleach intensities were
645 averaged and used for calculations using the formula $FRET_{\text{eff}} = (D_{\text{post}} - D_{\text{pre}}) / D_{\text{post}}$, where D is
646 intensity in arbitrary units.

647 ***Protein extraction and Immunoblotting***

648 Two hundred milligrams of leaf material were mixed with 350 μL of extraction buffer (100
649 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40 and 1x Protease inhibitor cocktail
650 (Sigma, P599)) and centrifuged for 15 min at 14,000 g. 4X Laemmli sample was added to 100
651 μL supernatant and boiled for 5 min. Equal amounts of supernatant were loaded on 10% poly-
652 acrylamide gels and blotted on a polyvinylidene difluoride (PVDF) membrane. α -Myc and α -
653 rabbit horseradish peroxidase conjugate (Amersham, GE Healthcare) were used at dilutions
654 1:1,000 and 1:5,000, respectively. The reaction was developed for 1 min using a Luminata
655 Crescendo Millipore immunoblotting detection system (Millipore, WBLUR0500).

656 For detection of phosphorylated forms of SnRK1 proteins, 10-day-old seedlings were collected
657 and ground in liquid nitrogen and the proteins were extracted using the following extraction
658 buffer: 25 mM Tris-HCl pH 7.8, 75 mM NaCl, 15 mM EGTA, 10 mM MgCl_2 , 10 mM B-
659 glycerophosphate, 15 mM 4-Nitrophenylphosphate bis, 1 mM DTT, 1 mM NaF, 0.5 mM
660 Na_3VO_4 , 0.5 mM PMSF, 1% Protease inhibitor cocktail (Sigma, P599), 0.1% Tween-20. The
661 protein extracts were centrifuged at 13,000 rpm and 4°C for 10 min and supernatants transferred
662 to a new tube. The protein concentration was measured using Bradford Dye Reagent (Bio-Rad);
663 equal amounts (15 μg) of total protein for each sample were separated by SDS-PAGE (10%
664 acrylamide gel) and transferred to a PVDF membrane (Bio-Rad). The membrane was blocked
665 in TBST buffer containing 5% (w/v) BSA and incubated with primary antibody and secondary
666 antibody. Antibodies used for immunoblotting were as follows: α -Phospho-AMPK α (Thr175)
667 (α -pT175) (1:1,000, Cell Signaling Technology), α -Kin10 (1:1,000, Agrisera), α -Kin11
668 (1:1,000, Agrisera), and α -Actin (1:10,000, Agrisera).

669

670

671 ***Co-immunoprecipitation (Co-IP)***

672 For Co-IP assays, total proteins from 7-day-old seedlings were extracted with no-salt lysis
673 buffer (50 mM Tris, pH 8.0, 0.1% Nonidet P-40, and 1% Protease inhibitor cocktail [Sigma])
674 at a fresh weight:buffer volume ratio of 1 g:2 mL. After centrifugation at 6,000 g and 4°C for
675 5 min, 20 μ L of α -GFP microbeads (Miltenyi Biotec) were added to the resultant supernatant
676 and incubated for 1 h at 4°C on a rotating wheel. Subsequent washing and elution steps were
677 performed according to the manufacturer (μ MACS GFP Isolation Kit; Miltenyi Biotec).
678 Immunoblot analysis was done essentially as described above, and immunoprecipitates from
679 transgenic lines expressing free GFP were used as controls. GFP-TSN-interacting proteins and
680 native TSN were detected by mouse α -GFP (monoclonal antibody JL-8; Clontech) and rabbit
681 α -TSN antibodies at final dilutions of 1:1,000 and 1:5,000, respectively.

682

683 ***Image Analysis***

684 The image analysis was done using ImageJ v1.41 (NIH) software
685 (<http://rsb.info.nih.gov/ij/index.html>). Co-localization analyses were performed as described
686 previously (French et al., 2008) using Pearson (rp) and Sparman (rs) statistics.

687

688 ***Bioinformatics***

689 Functional annotation of Gene Ontology was performed using Panther (Mi et al., 2017).
690 Information about subcellular localization for all proteins was performed using SUBA4 (Tanz
691 et al., 2013). Prion-like domains were identified using the web application PLAAC (Lancaster
692 et al., 2014). The selected parameters were as follow: minimum length for prion domains was
693 60 (Lcore =60); organism background was *Arabidopsis*; and the α parameter was 1 (α =1). The
694 RNA-binding proteins were predicted by the RNAPred tool (Kumar et al., 2011). The selected
695 parameters were as follows: prediction approach was amino acid composition and threshold for
696 the support vector machine (SVM) was 0.5. To build protein-protein interaction networks we
697 used STRING database (Jensen et al., 2009). We used Cytoscape to visualize the resulting PPI
698 dataset (Gagneur et al., 2006). Per-residue disorder content was evaluated by PONDR
699 predictors, including PONDR-FIT (Xue et al., 2010) and PONDR-VSL2 (Peng et al., 2005).
700 The intrinsic disorder propensities of TSN were evaluated according to the method described
701 by Uversky et al. (2017) (Santamaria et al., 2017; Uversky, 2017). Disorder evaluations
702 together with disorder-related functional information were retrieved from the D₂P₂ database
703 (<http://d2p2.pro/>) (Oates et al., 2013). LLPS predisposition was evaluated using the PSPredictor
704 tool (Sun. et al., 2019).

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711 AUTHORS' CONTRIBUTIONS

712 Conceptualization, E.G.-B.; Methodology, E.G.-B., and P.V.B.; Investigation, E.G.-B., P.H.E.,
713 V.U., and K.D.; Writing – Original Draft, E.G.-B.; Writing – Review & Editing, E.G.-B.,
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715

716 FIGURE LEGENDS

717

718 **Figure 1. Identification of *Arabidopsis* TSN-interacting proteins by alternative tandem**
719 **affinity purification (TAPa).** *A*, Schematic illustration of the expression cassette in TAPa
720 vector. The vector allows translational fusion of TSN and GFP at their C-termini to the TAPa
721 tag. The expression is driven by two copies of the cauliflower mosaic virus 35S promoter
722 (2x35S) and a tobacco mosaic virus (TMV) U1 X translational enhancer. The TAPa tag consists
723 of two copies of the protein A IgG-binding domain (IgG-BD), an eight amino acid sequence
724 corresponding to the 3C protease cleavage site (3C), a 6-histidine stretch (His), and nine repeats
725 of the Myc epitope (myc). A Nos terminator (Nos ter) sequence is located downstream of each
726 expression cassette. *B*, Immunolocalization of TSN2-TAPa and GFP-TAPa fusion proteins in
727 root cells of 5-day-old seedling. The seedlings were grown under no stress conditions (23°C)
728 or incubated for 40 min at 39°C (heat stress) and then immunostained with α -Myc. Scale bars
729 = 10 μ m. *C*, Schematic representation of three classes of proteins found upon comparative
730 analysis of TSN interactomes isolated under no stress and heat stress conditions.

731

732 **Figure 2. Proteomic analysis of the *Arabidopsis* TSN2 interactomes.** *A*, Venn diagram
733 showing comparison of TSN2_HS interactome with human and yeast SG proteomes. *B*,
734 Frequency of RBPs and proteins with prion-like domain or ATPase activity found among
735 TSN2_NS and TSN2_HS protein pools in comparison with yeast and human SG proteomes. *C*,
736 Venn diagram showing comparison between TSN2_NS and TSN2_HS protein pools.

737 Classification of TSN2-interacting proteins in three classes: HS-sensitive, HS-independent and
738 HS-dependent. Within each class the proteins are further classified into two groups, based on
739 their known localization to SGs in non-plant (Group 1) or plant (Group 2) organisms. **D**,
740 Network maps showing physical interactions among proteins in the TSN2_NS and TSN2_HS
741 pools.

742
743 **Figure 3. TSN2 interactomes are enriched in IDR-containing proteins.** **A**, Per-protein
744 propensities for disorder (average of the corresponding per-residue propensities) evaluated by
745 PONDR-FIT (*x*-axis) and by PONDR-VSL2 (*y*-axis) for TSN2_NS and TSN2_HS
746 interactomes. **B**, Classification of proteins from the whole *Arabidopsis* proteome and TSN2_NS
747 and TSN2_HS interactomes based on their ID content. **C**, Correlation between the ID content
748 evaluated by PONDR-FIT (*y*-axis) and predisposition to undergo LLPS (*x*-axis). **D**, Disorder
749 profiles of TSN2 generated by PONDR-VLXT, PONDR-VL3, PONDR-VSL2, IUPred-short,
750 IUPred-long and PONDR-FIT and a consensus disorder profile (based on mean values of six
751 predictors). SN, staphylococcal nuclease domain.

752
753 **Figure 4. TSN2 and its interactors are localized in heat-induced SGs.** **A**, List of TSN-
754 interacting proteins included in the co-localization analysis. **B**, Co-localization of RFP-TSN2
755 (red) with GFP-ADH2 (negative control) and GFP-UBP1 (positive control) in *N. benthamiana*
756 protoplasts incubated under control conditions (23°C) or at 39°C for 30 min (HS). Scale bars =
757 5 μm. **C**, Pearson and Spearman coefficients (r_p and r_s , respectively) of co-localization (PSC)
758 of RFP-TSN2 with individual GFP-tagged TSN-interacting proteins listed in **A** and with both
759 negative and positive control proteins (denoted by red arrowheads) under HS. **D**, BiFC between
760 cYFP-TSN2 and nYFP-TSN-interacting proteins in *N. benthamiana* protoplasts after HS (39°C
761 for 30 min). Scale bars = 5 μm. **E**, Schematic diagram of TSN protein domain organization
762 depicting SN and Tudor regions. **F**, BiFC between cYFP-TSN2 (full-length), cYFP-SN or
763 cYFP-Tudor and nYFP-TSN-interacting proteins in *N. benthamiana* protoplasts after HS (39°C
764 for 30 min). Scale bars = 10 μm. Chart shows quantification of the reconstituted YFP signal.
765 AU, arbitrary units. Data are means ± SE of BiFC signal level measured in three independent
766 experiments each containing seven individual measurements. Asterisks indicate significantly
767 different fluorescence intensity compared with the corresponding Tudor ($p < 0.05$, ANOVA).

768
769 **Figure 5. Proteomes of *Arabidopsis* TSN1 and TSN2 largely overlap.** **A**, Venn diagram
770 showing overlap between TSN1 and TSN2 interactomes isolated by TAPa from *Arabidopsis*

771 plants grown under no stress (NS) conditions. Common interactors of TSN1 and TSN2 are
772 classified into three groups. **B**, Co-IP of TSN isoforms and RH12 in protein extracts prepared
773 from *N. benthamiana* leaves agro-infiltrated with GFP-TSN1 or GFP-TSN2 and Myc-RH12.
774 GFP was used as a negative control. Co-IP was analysed by immunoblotting using α -Myc and
775 α -GFP. **C**, Localization of RH12 in root cells of 5-old-day *Arabidopsis* seedlings expressing
776 GFP-RH12 under control of the native promoter. The seedlings were grown under 23°C (NS)
777 or incubated at 39°C for 40 min (HS). Scale bars = 10 μ m.

778

779 **Figure 6. Characterization of presumed *Arabidopsis* SG core proteins.** **A**, Venn diagram
780 showing comparison between TSN2_NS and TSN2_NaCl pools. Common TSN2 interactors
781 are classified into two groups, based on their known localization to SGs in non-plant (Group 1)
782 or plant (Group 2) organisms. **B**, Venn diagram showing comparison between TSN2_NS,
783 TSN2_HS and TSN2_NaCl pools. Eleven common TSN2 interactors are ascribed as presumed
784 core SG components. **C**, Co-IP of TSN and Rbp47 in protein extracts prepared from
785 *Arabidopsis* seedlings expressing Pro35S:GFP-Rbp47 and grown under no stress (C; NS), HS
786 (39°C for 40 min) or salt stress (150 mM NaCl for 40 min) conditions. The GFP-expressing
787 line was used as a negative control. Endogenous TSN (107 KDa) was detected in total fractions
788 (Input) and fractions co-immunoprecipitated (Co-IP) with Rbp47 but not with free GFP in all
789 three conditions. Co-IP was analysed by immunoblotting using α -TSN and α -GFP. **D**,
790 Localization of Pro35S:GFP-Rbp47, Pro35S:GFP-UBP1, Pro35S:GFP-TCTP and
791 Pro35S:GFP-SnRK1.2 in root cells of 5-old-day *Arabidopsis* seedlings. The seedlings were
792 grown under 23°C (NS) or incubated at 39°C for 40 min (HS) or with 150 mM NaCl for 40 min
793 (NaCl). Scale bars = 10 μ m

794

795 **Figure 7. Activation of SnRK1 under HS depends on the presence of both TSN and SGs.**
796 **A**, Co-IP of TSN and SnRK1.2 in protein extracts prepared from *Arabidopsis* seedlings
797 expressing Pro35S:GFP-SnRK1.2 and grown under heat stress condition (39°C for 40 min).
798 The GFP-expressing line was used as a negative control. Endogenous TSN (107 KDa) was
799 detected in the total fractions (Input) and in the fraction co-immunoprecipitated (Co-IP) with
800 SnRK1.2 but not with free GFP. Co-IP was analysed by immunoblotting using α -TSN and α -
801 GFP. **B**, FRET assay of the indicated protein combinations using CFP-YFP pair in *N.*
802 *benthamiana* leaves under HS (39°C for 40 min). EV, empty vector (negative control). Data
803 show mean \pm SD of 10 replicate measurements. The experiment was repeated three times with
804 similar results. **C-F**, Immunoblot analysis with α -SnRK1.1, α -SnRK1.2, α -p-T175 and α -Actin

805 of protein extracts prepared from root tips of 10-day-old *Arabidopsis* Col (**C**, **D**), *tsn1tsn2* (**E**)
806 or *tsn1tsn2* expressing *ProTSN2:GFP-TSN2* (**F**) seedlings heat-stressed (39°C) for 0, 20, 40
807 and 60 min. **D**, The seedlings were pre-treated with cycloheximide (CHX, 200 ng/μL) and then
808 subjected to HS. SnRK1 activity was determined as the ratio of phosphorylated to total SnRK1
809 protein. The data on charts in **C-F** show mean ratio of phosphorylated to total SnRK1 (both
810 isoforms) integrated band intensity level ± SD from five experiments. Asterisks denote
811 significant difference; Student's t test, $P < 0.05$.

812
813 **Figure 8. Schematic drawing showing maturation of SGs upon stress perception**
814 **whereupon TSN serves a docking platform for SG components.** Plant stress granules can
815 be hypothesized to undergo two phases of assembly. The first phase of this model is formation
816 of a pre-assembly state in which stress granules core is assembled by protein-protein interaction
817 mediated by IDR from proteins present in a pre-existing TSN-SG complex. Second, formation
818 of a larger microscopically visible stress granules (mature state) is constituted upon removing
819 of stress-sensitive TSN interactors and incorporation of stress-dependent interactors. Dashed
820 lines represent physical interactions between IDR-containing proteins. Red lines represent
821 possible sites of activity of the ATP modulators. The upper part show 5-day-old Col seedlings
822 expressing *ProTSN2:TSN2-GFP* grown under no stress (23°C, Basal state) or incubated for 40
823 min at 39°C (Stressed state).

824
825 **Figure S1. Transgenic *Arabidopsis* lines used in this study.** **A**, Expression of TSN2-TAPa
826 (167 KDa) and GFP-TAPa (83 KDa) and **B**, TSN1-TAPa (170 KDa) in Col background
827 confirmed by immunoblotting with α-Myc.

828
829 **Figure S2: Schematic representation of the TAPa purification procedure.** During the first
830 affinity purification step, plant protein extracts are incubated with IgG beads followed by
831 elution through the specific cleavage of TAPa tag with the low-temperature active rhinovirus
832 3C protease. At the second affinity purification step, IgG bead eluates are incubated with Ni
833 beads followed by the elution of proteins from beads using imidazole-containing buffer.

834
835 **Figure S3. Immunoblotting of crude protein extracts (Input) and purified protein**
836 **fractions (Ip) obtained during small-scale TAPa purification.** TSN2-TAPa and GFP-TAPa
837 (**A**) and TSN1-TAPa (**B**) fusion proteins were detected using α-Myc.

838

839 **Figure S4. Flow chart of proteomics data generation.**

840

841 **Figure S5. Venn diagrams.** (A) Comparison between mammalian and yeast SG proteomes
842 with TSN2_NS interactome. (B), Comparison between TSN2_NS, TSN2_HS and Rbp47b
843 proteomes.

844

845 **Figure S6. Analysis of the whole proteome of *Arabidopsis* for ID.** Per-protein propensities
846 for disorder (average of the corresponding per-residue propensities) evaluated by PONDR-FIT
847 (x-axis) and by PONDR-VSL2 (y-axis).

848

849 **Figure S7. Prevalence and functionality of ID in the *Arabidopsis* TSN proteins.** A,
850 Evaluation of the functional ID propensity by the D2P2 database. In the corresponding plot, top
851 nine colored bars represent the location of IDRs predicted by different disorder predictors
852 (Espritz-D, Espritz-N, Espritz-X, IUPred-L, IUPred-S, PV2, PrDOS, PONDRs VSL2b, and
853 PONDRs-VLXT, see keys for the corresponding color codes. Green/Blue-and-white bar in the
854 middle of the plot shows the predicted disorder agreement between these nine predictors, with
855 green/blue parts corresponding to IDRs by consensus. The yellow bar shows the location of the
856 predicted disorder-based binding site (MoRF region). B, Evaluation of the per-residue disorder
857 propensity of TSN1 using six different disorder predictors, and a consensus disorder profile
858 (based on mean values of six predictors). SN, staphylococcal nuclease domain.

859

860 **Figure S8. Co-localization of GFP-TSN2-interacting proteins (green) and RFP-TSN2**
861 **(red) quantified in Figure 4C.** Insets show enlarged boxed areas. Scale bars = 5 μ m.

862

863 **Figure S9. BiFC between TSN2 and TSN2-interacting proteins under no stress (NS)**
864 **condition.** BiFC between cYFP-TSN2 and nYFP-TSN-interacting proteins (TIPs) in *N.*
865 *benthamiana* protoplasts incubated at 23°C. BiFC analysis of cYFP-TSN2 and nYFP-TIPs
866 (only one representative example is shown) with empty vectors encoding nYFP and cYFP,
867 respectively was used as a negative control. Scale bars = 5 μ m.

868

869 **Figure S10. Activation of SnRK1 isoforms under ABA treatment.** A, Localization of
870 SnRK1.2 is shown in the root cells of 5-day-old Col seedlings expressing Pro35S:GFP-
871 SnRK1.2. The seedlings were grown under control conditions or incubated for 40 min at 10 μ M
872 of ABA (ABA). Bars = 10 μ m. B, Immunoblot analysis using α -SnRK1.1, α -SnRK1.2, α -p-

873 T175 and α -Actin in ABA-stressed 10-day-old *Arabidopsis* Col seedlings for 0, 20, 40 and 60
874 min. C, SnRK1 activity was determined as the ratio of phosphorylated to total SnRK1 protein.
875 Three biological replicates were analysed for quantification. Asterisks denote significant
876 difference; Student's t-test, $P < 0.05$.

877

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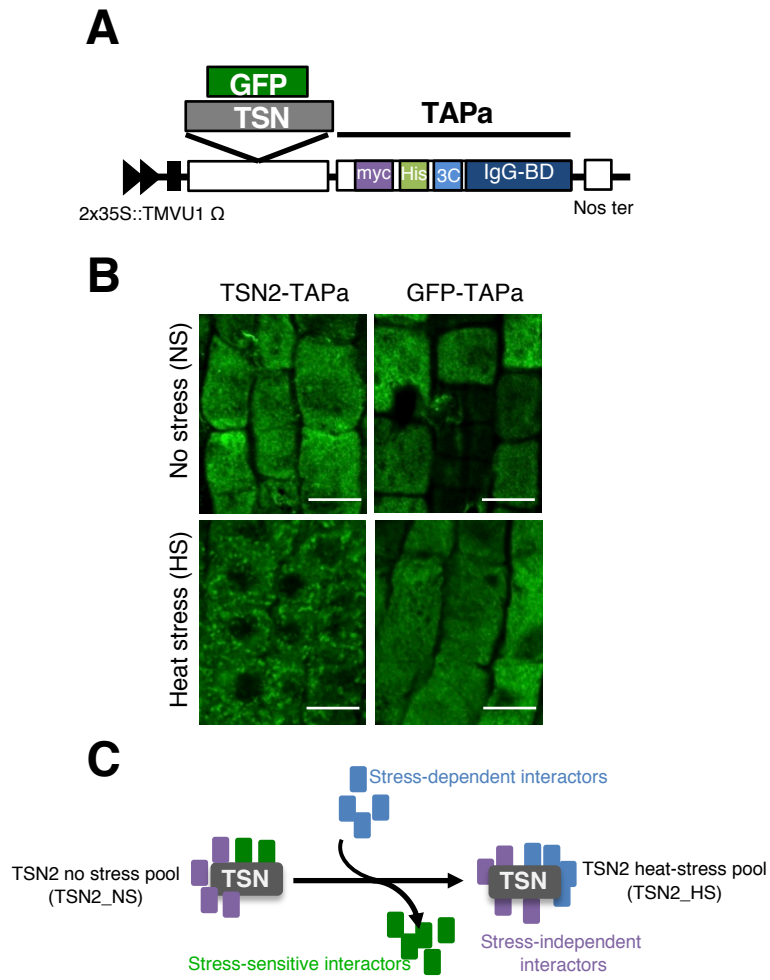


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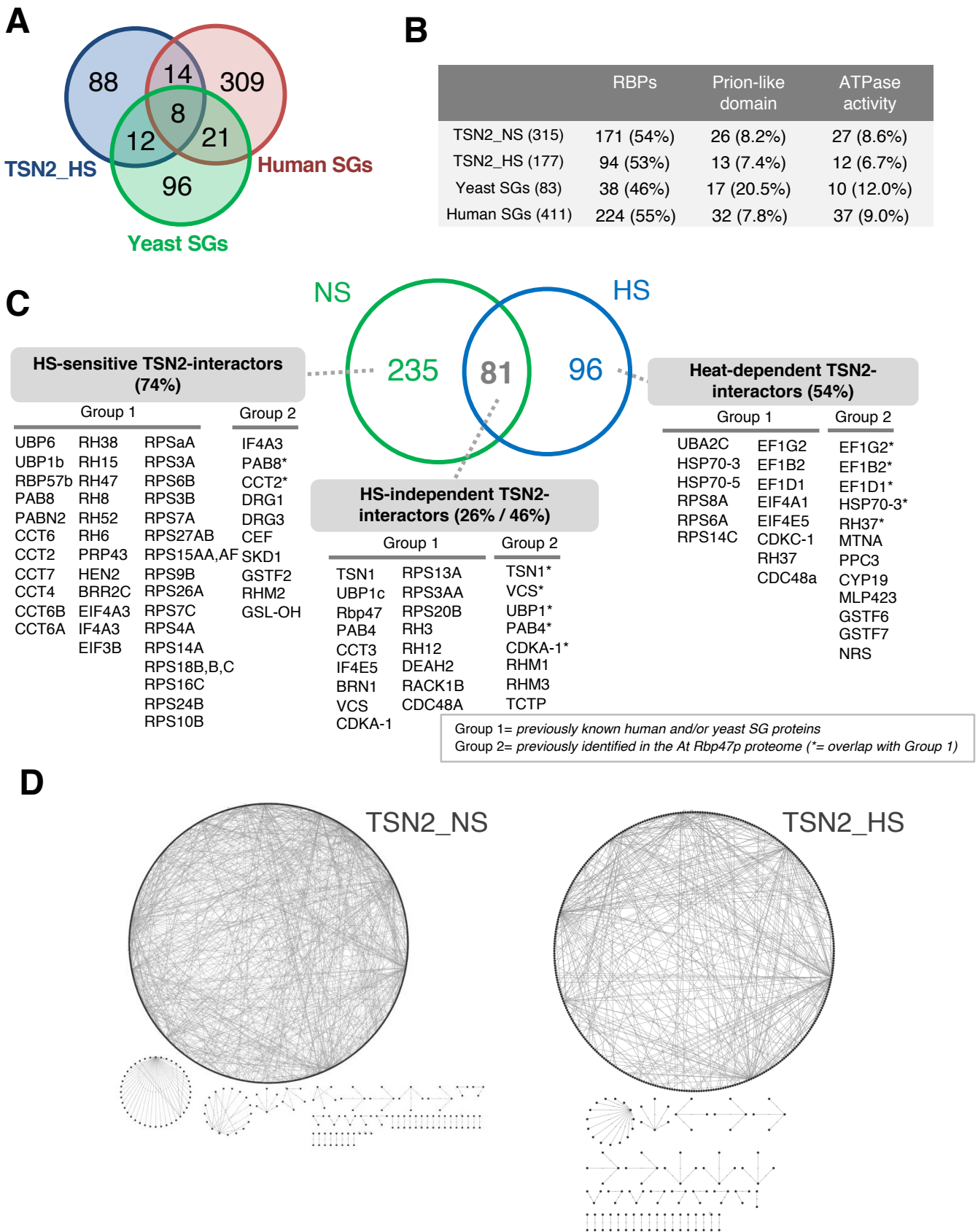


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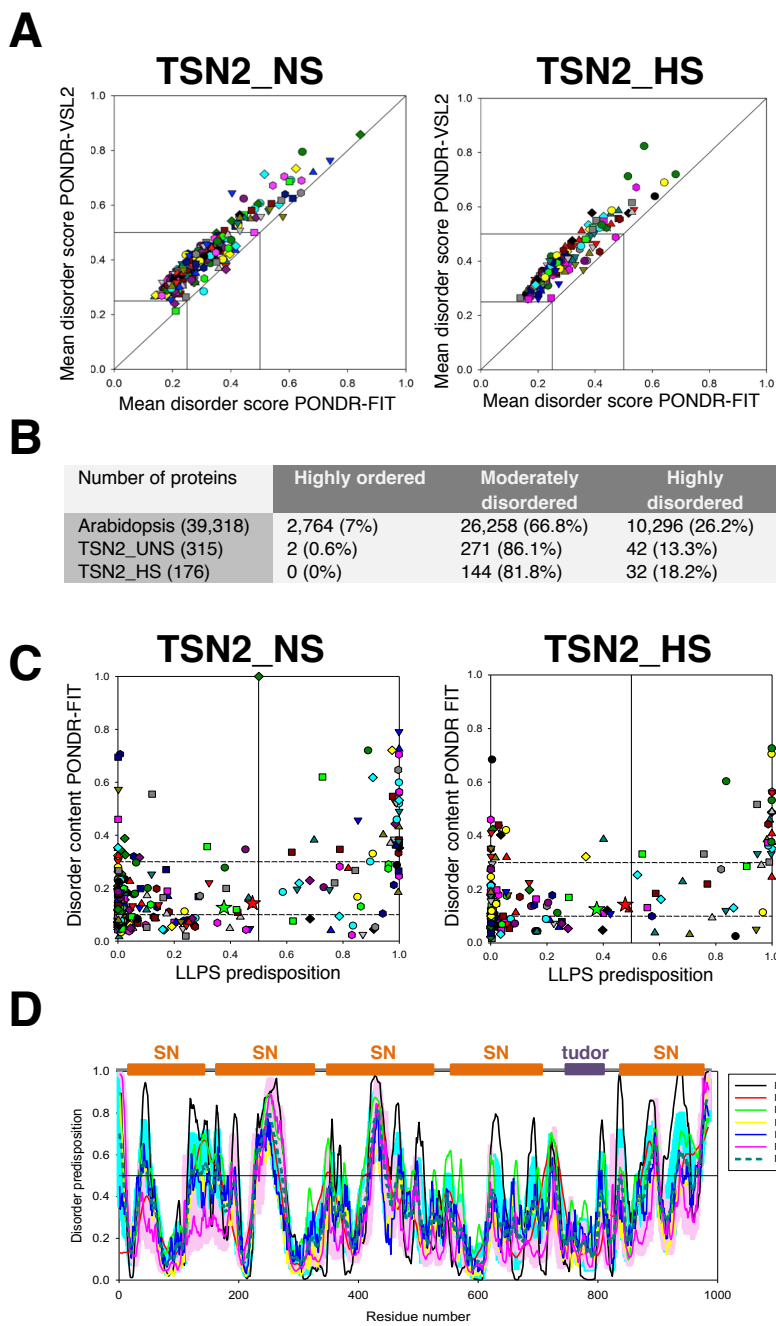


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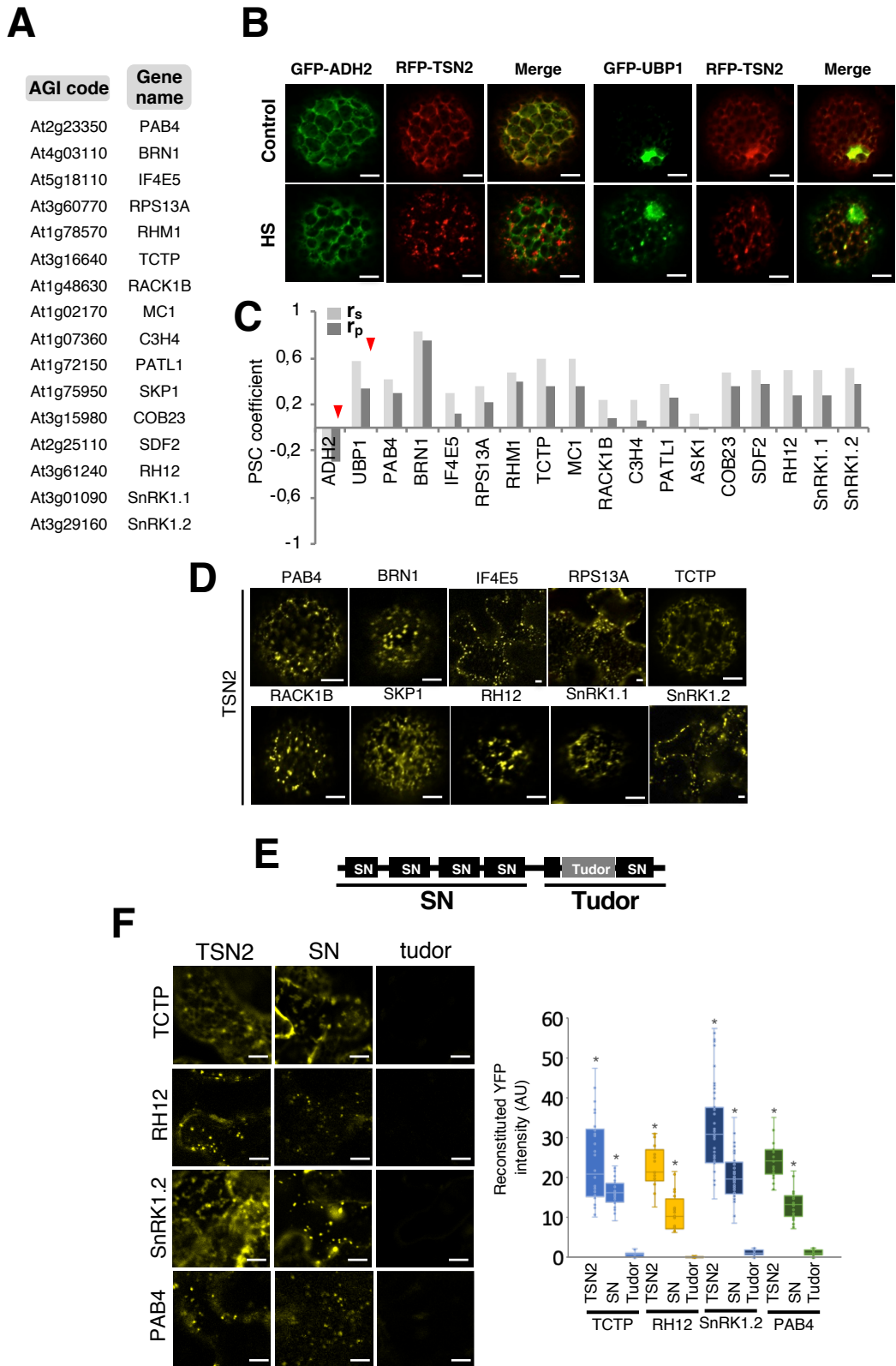
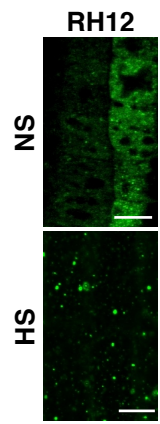
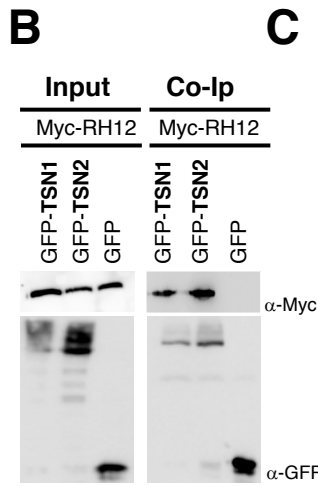
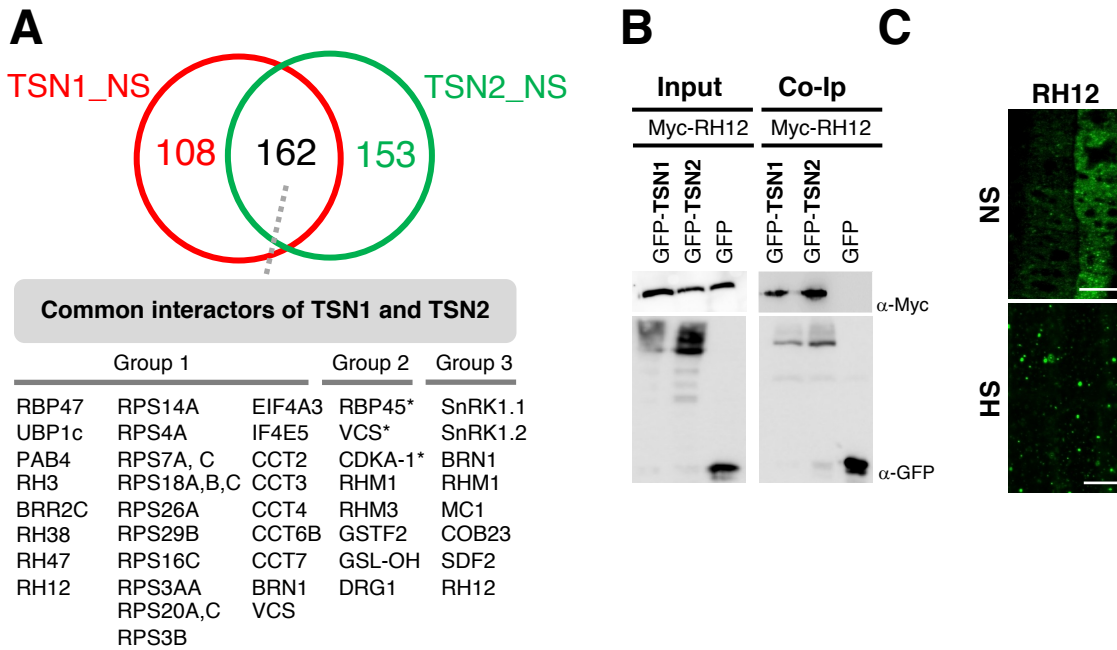


Figure 4.



Group 1= previously known human and/or yeast SG proteins
 Group 2= previously identified in the *At Rbp47p* proteome (*= overlap with Group 1)
 Group 3= novel plant SG components analysed in the Figure 4

Figure 5.

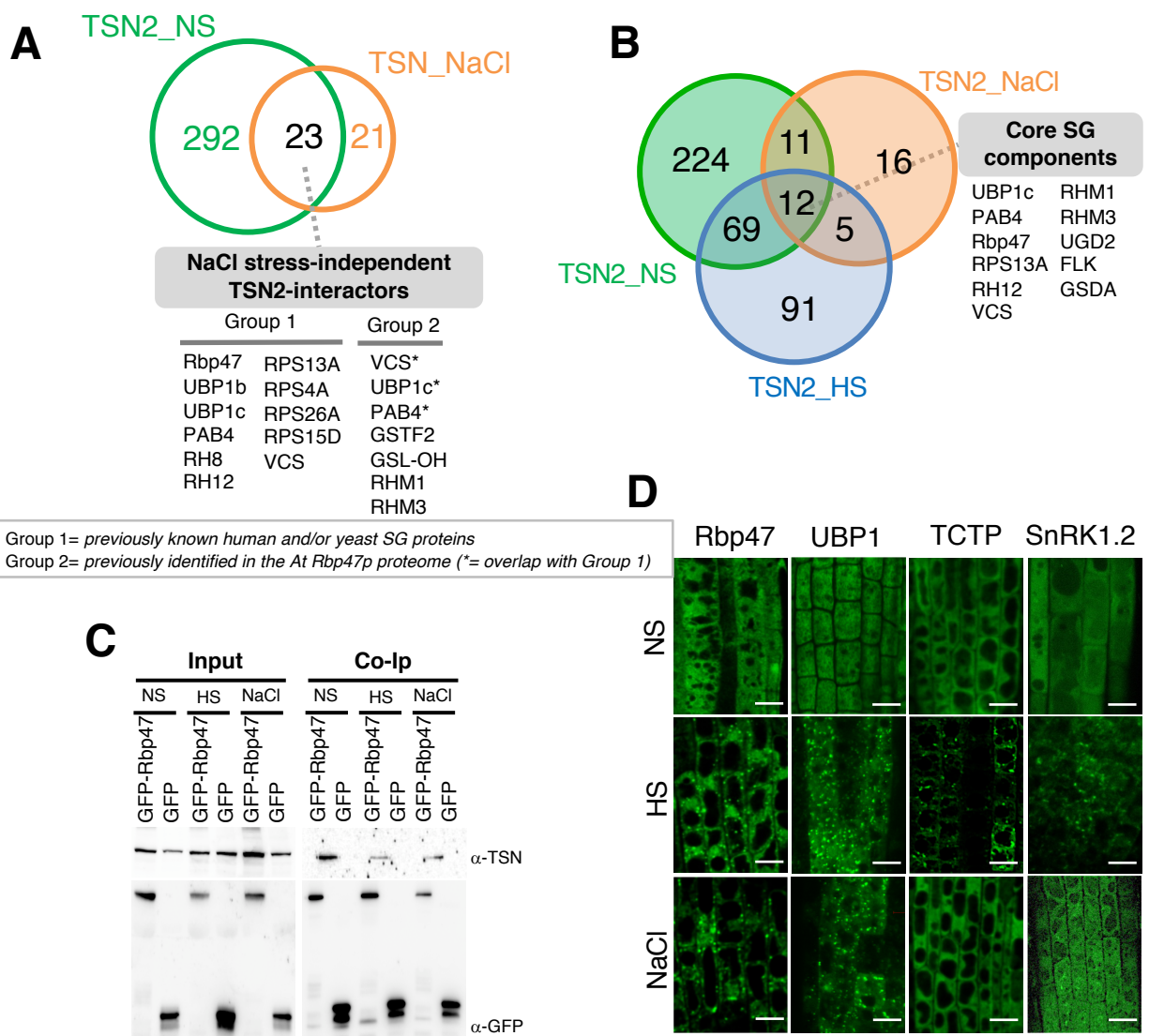


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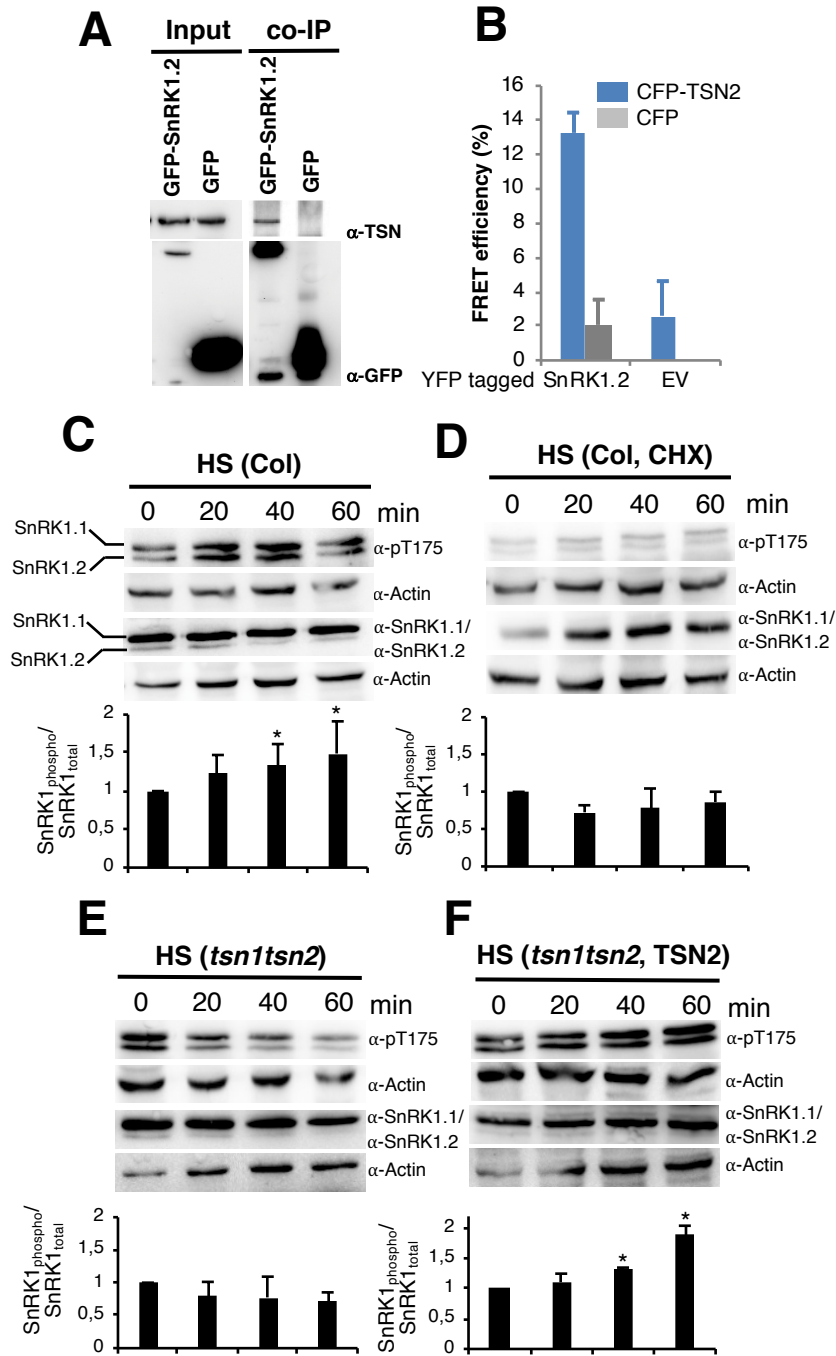


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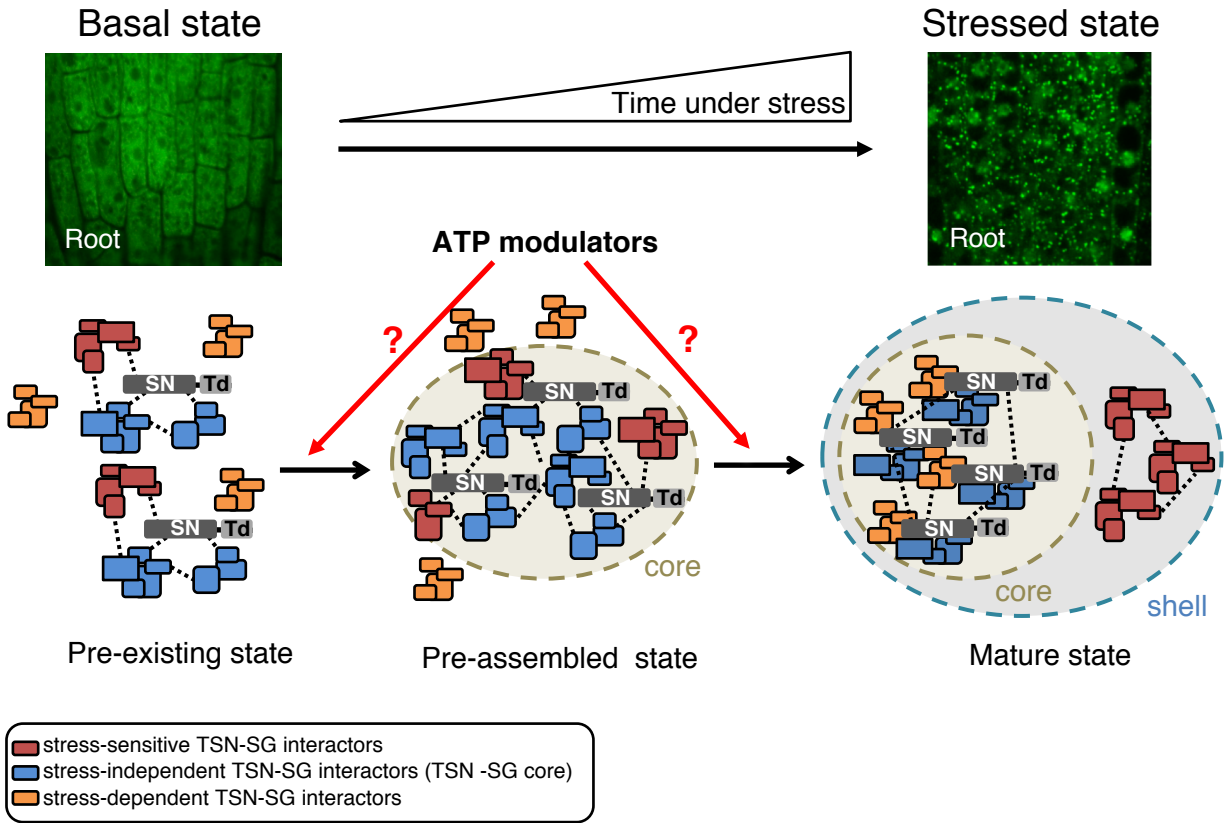
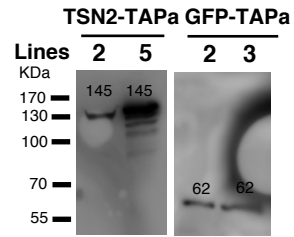


Figure 8.

A



B

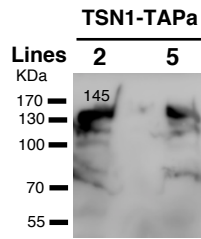


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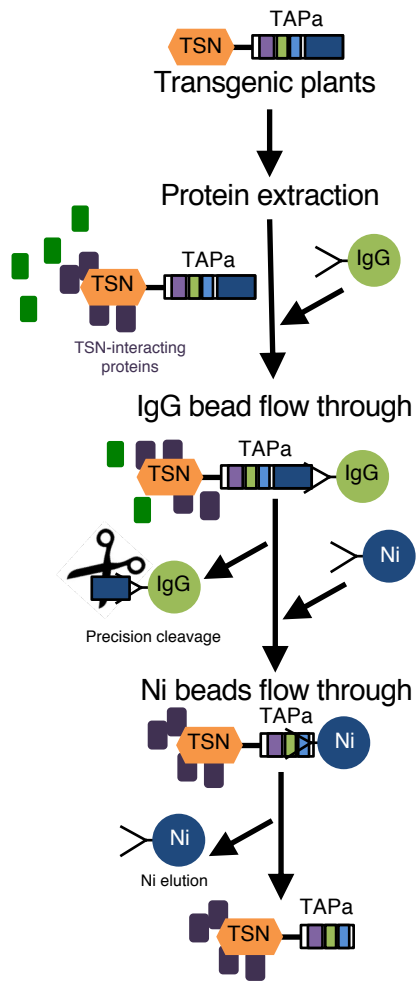


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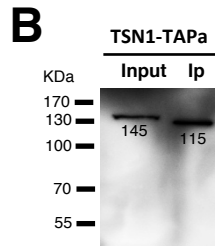
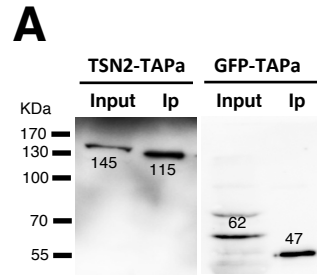


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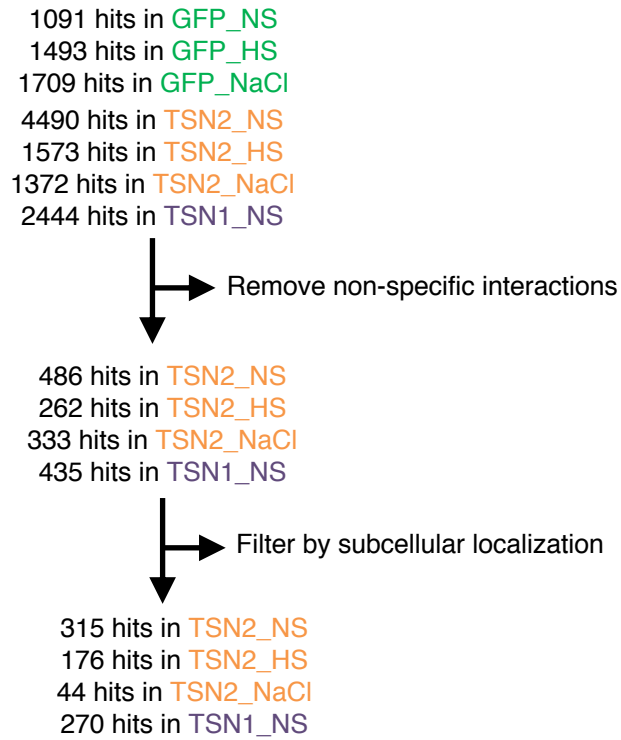
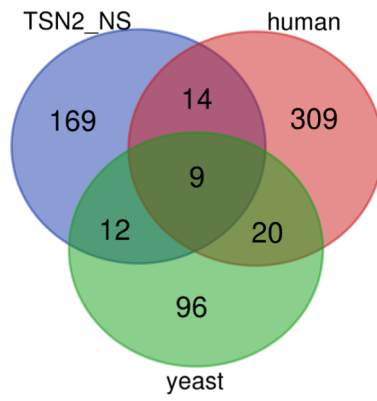


Figure S4.

A



B

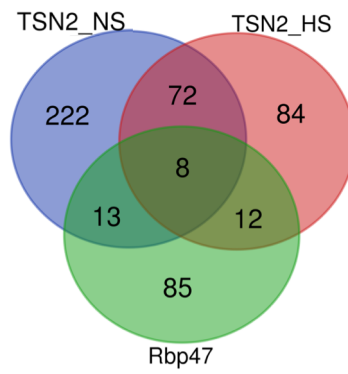


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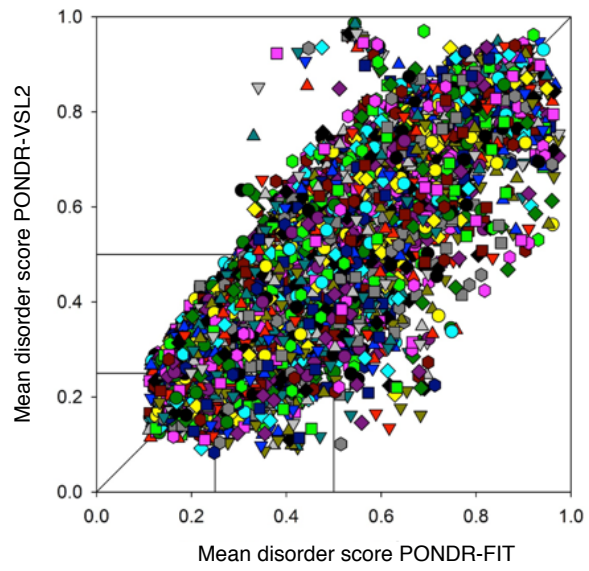
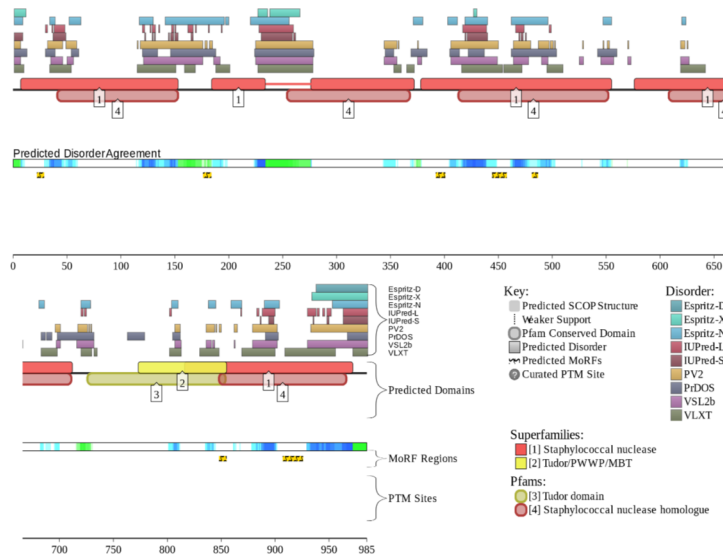
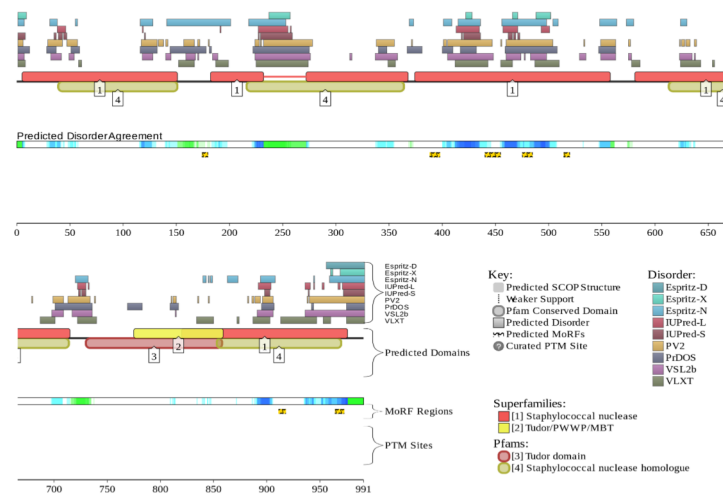
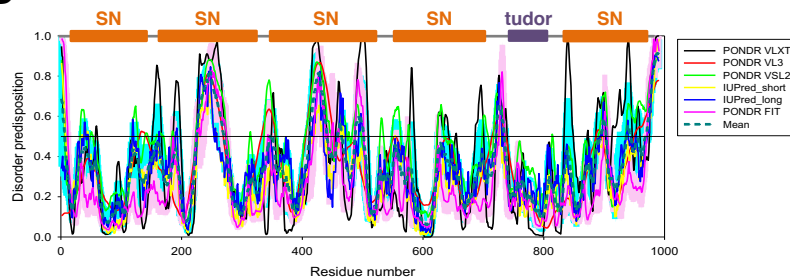
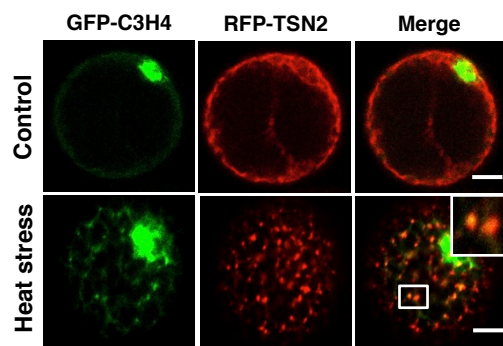
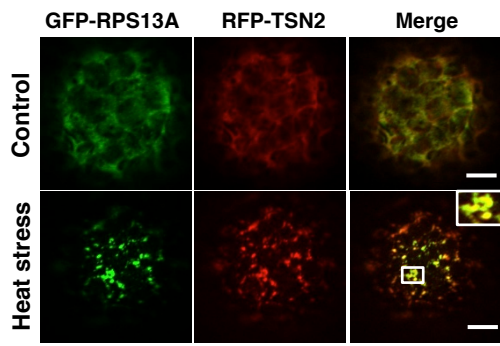
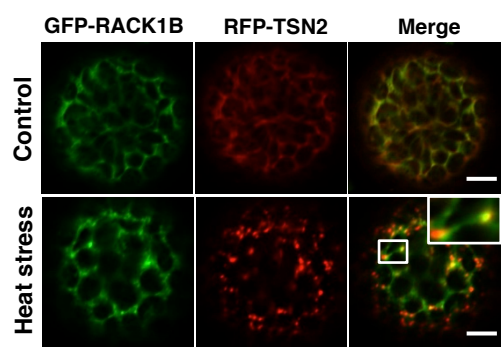
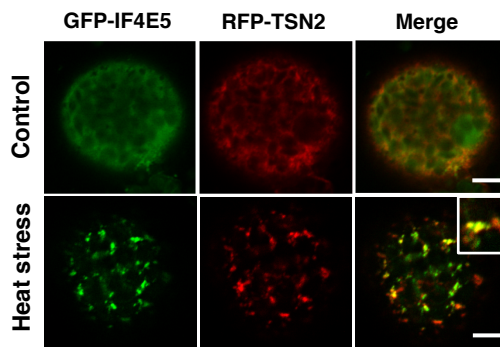
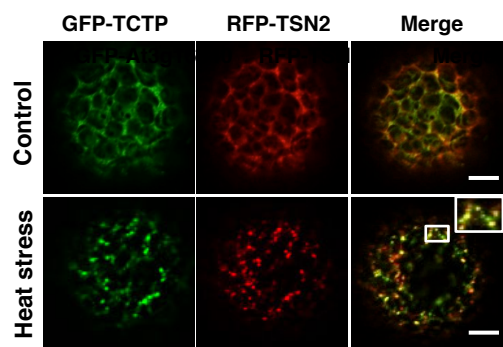
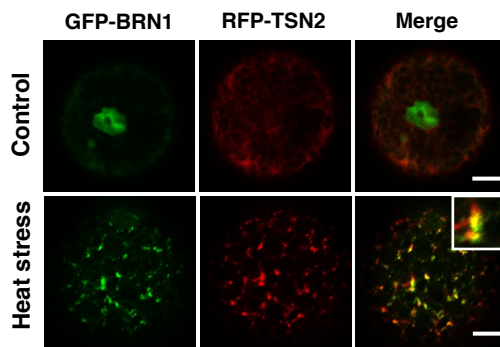
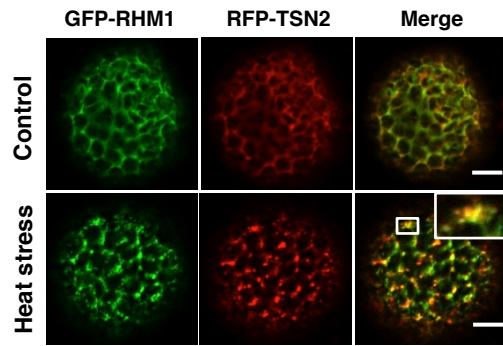
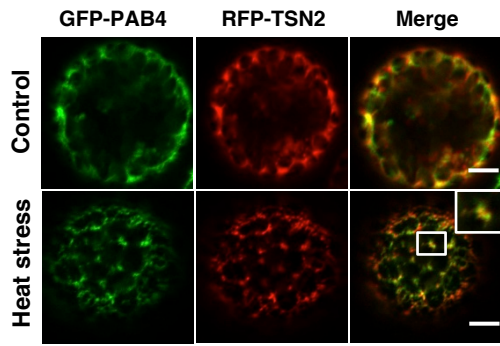


Figure S6.

A**TSN2****TSN1****B****Figure S7.**



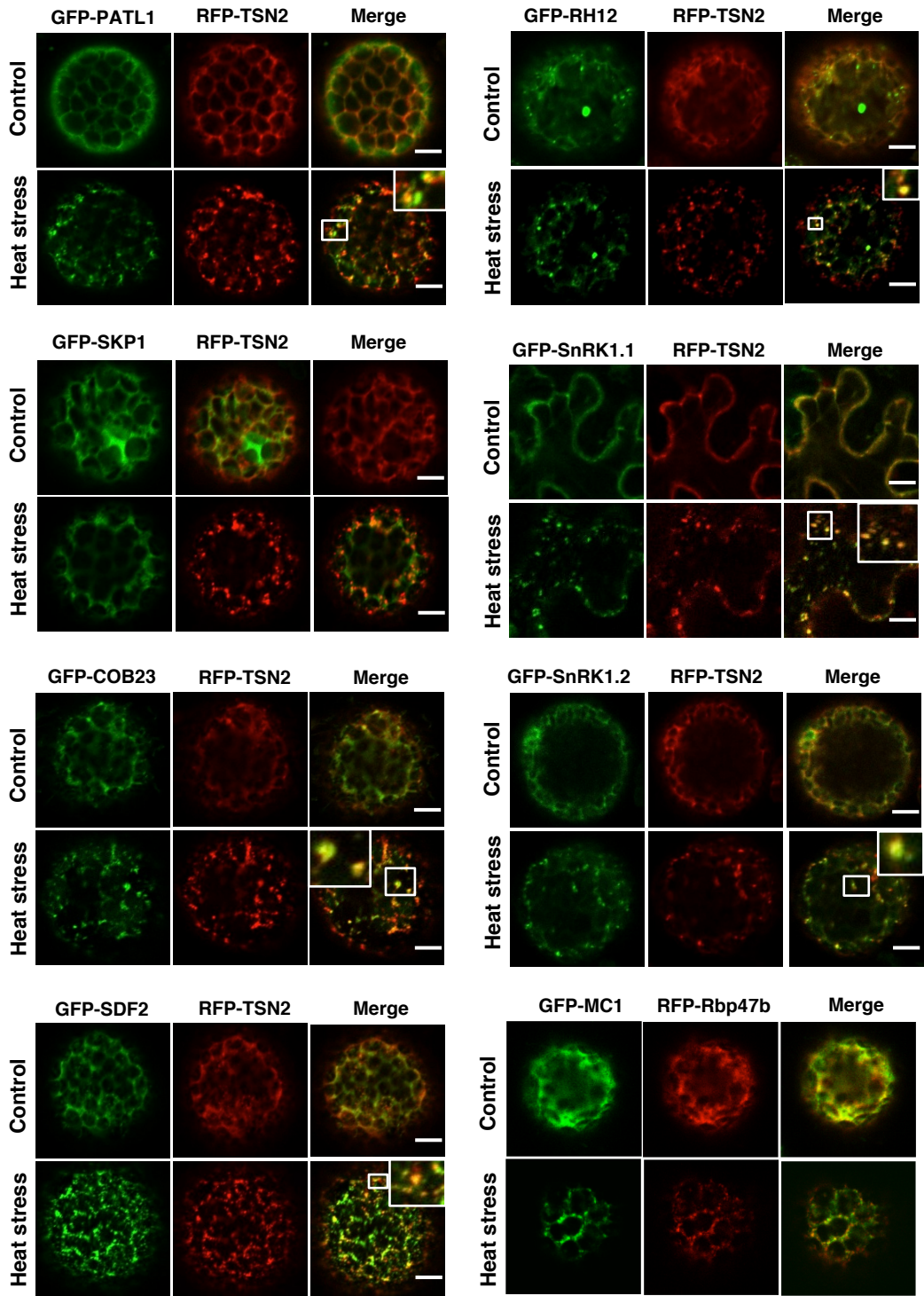


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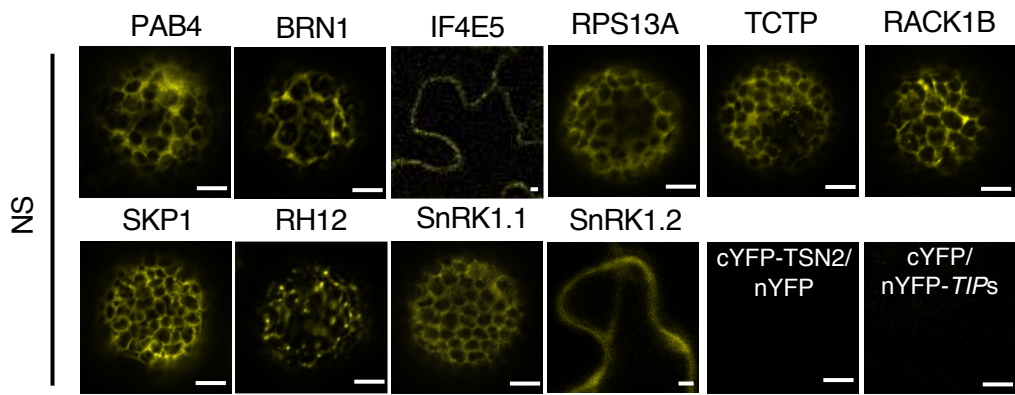


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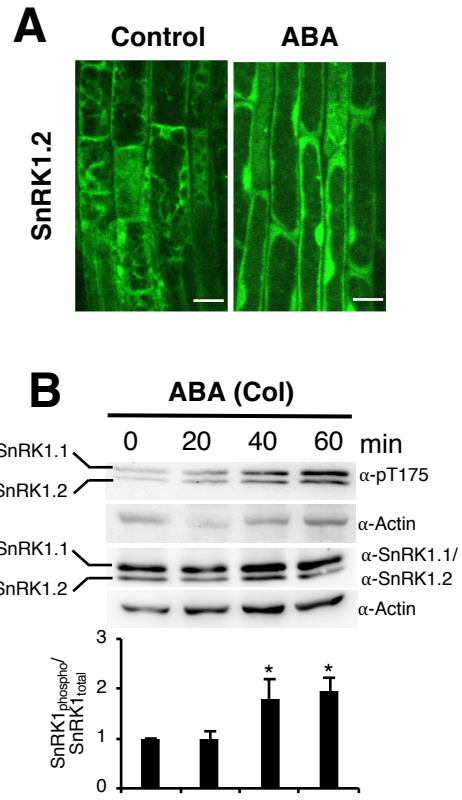


Figure S10.