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Seedlings Lacking the PTM Protein Do Not Show a *genomes uncoupled* (*gun*) Mutant Phenotype[1[OPEN]]

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Chloroplast development requires communication between the nucleus and the developing chloroplast to ensure that this process is optimized (Jarvis and López-Juez, 2013; Chan et al., 2016). This is especially true during de-etiolation as mis-regulation of chloroplast development can lead to seedling death from photooxidative damage. Retrograde signaling from the developing chloroplast (plastid) to the nucleus, which is termed biogenic signaling (Pogson et al., 2008), can be revealed using either the bleaching herbicide Norflurazon (NF), an inhibitor of carotenoid synthesis, or the plastid translation inhibitor, lincomycin (Lin) to damage the plastid. Under these conditions there is a strong downregulation of hundreds of nuclear genes (Koussevitzky et al., 2007; Aluru et al., 2009; Page et al., 2017). Despite decades of research, the biogenic retrograde signaling pathway is still very poorly understood. What we do know has mostly come from an innovative screen by the group of Joanne Chory in which *genomes uncoupled* (*gun*) mutants were identified that retained nuclear gene expression of chloroplast-related genes after NF treatment (Susek et al., 1993). This screen now defines the *gun* phenotype: increased expression, compared to wild type, of nuclear genes following chloroplast damage. In total, six original *gun* mutants have been described. GUN1 is a pentatricopeptide repeat protein with a still unknown function (Koussevitzky et al., 2007). The other GUNs are all related to the tetrapyrrole pathway (Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011). Further analysis of these mutants has supported the idea that tetrapyrroles are important for plastid signaling (Vinti et al., 2000; Strand et al., 2003; Moulin et al., 2008; Mochizuki et al., 2008; Voigt et al., 2010), and our current understanding is that the synthesis of heme by ferrochelatase 1 results in a positive signal that promotes expression of nuclear-encoded chloroplast genes (Woodson et al., 2011; Terry and Smith, 2013).

Additional mutants identified through screens for a *gun* phenotype are the blue-light photoreceptor mutant *cry1* (Ruckle et al., 2007) and the *coe1* mutant lacking a functional mitochondrial transcription termination factor 4 (Sun et al., 2016). A number of *happy on norflurazon* (*hon*) mutants were also identified by screening seedlings grown on NF under lower light intensities (Saini et al., 2011). This identified one *hon* mutation in the ClpR4 subunit of the chloroplast-localized Clp protease complex (Saini et al., 2011). Other mutants with a *gun* phenotype have been identified *via* informed approaches to test potential signaling components. These include the transcription factor mutants *abi4* (Koussevitzky et al., 2007), *hy5* (Ruckle et al., 2007), and *glk1glk2* (Waters et al., 2009). Interestingly, *GOLDEN2-LIKE* (*GLK*) overexpressing plants (Leister and Kleine, 2016) have also been reported to show *gun* phenotypes, perhaps reflecting the complex relationship between the anterograde signals by which the nucleus controls chloroplast development and retrograde signaling (Martin et al., 2016).

In 2011, Sun et al. identified a PHD transcription factor associated with the chloroplast envelope, called PTM, which they proposed mediates chloroplast signals to the nucleus through cleavage in response to changes in plastid status. Accumulation of the N terminus of the protein in the nucleus would then inhibit nuclear gene expression. Consistent with this, they reported that

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the *ptm* mutant has a *gun* phenotype with elevated expression compared to wild type of *Lhcb* on both NF and Lin. This was a significant result for the field as it defined a mechanism for plastid signaling, and is unsurprisingly included in numerous models for this pathway (e.g. Chan et al., 2016; Bobik and Burch-Smith, 2015; Terry and Smith, 2013; Barajas-López et al., 2013). Subsequent studies from the same group have suggested that PTM functions in retrograde signaling from the chloroplast to regulate flowering under high light (Feng et al., 2016) and in the integration of light and chloroplast retrograde signaling during de-etiolation (Xu et al., 2016). However, the demonstration that PTM shows a *gun* phenotype and is involved in retrograde signaling has yet to be supported by additional experimental data from other groups.

Given the potential importance of PTM for our understanding of plastid signaling, we have further examined the role of PTM in responses to NF and Lin in two different laboratories (See Supplemental Materials and Methods and Table S1). For the experiments at Southampton, it was necessary for us to isolate the same insertional *ptm* mutant allele described in Sun et al. (2011) from the SALK collection because this was no longer available from the authors. Isolation of the *ptm* mutant for this study, which we name here as *ptm-1*, is described in Supplemental Figure S1. Analysis of gene expression after NF treatment was then performed. As shown in Figure 1A, 5 μM NF treatment using the experimental conditions (1% Suc, 25 μmol m^{-2} s^{-1} white light (WL) for 7 d) of Woodson et al. (2011) resulted in no change in gene expression for a suite of five photosynthesis-related genes (including *LHCB2.1* used by Sun et al. (2011) for their real-time PCR experiments) in *ptm-1* compared to wild-type seedlings, whereas there was clear rescue of gene expression in the control *gun5* and *gun6* mutants. Next we repeated the

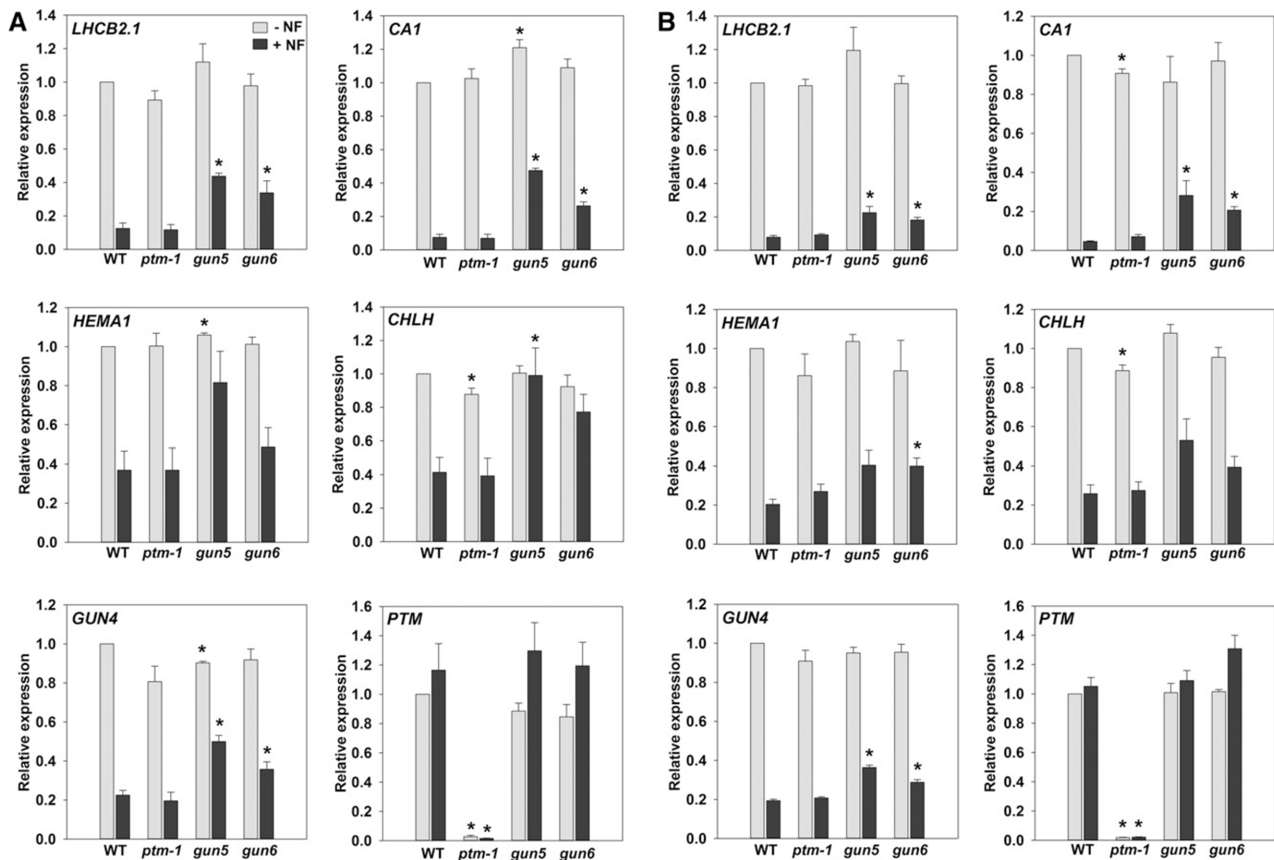

Figure 1. The *ptm-1* mutant does not show a *gun* phenotype on Norflurazon (NF). Seedlings were grown on half-strength Linsmaier and Skoog medium (A) supplemented with 1% Suc and 0.8% agar (pH 5.7) with (dark gray bars) or without (light gray bars) 5 μM NF under continuous low white light (25 μmol m^{-2} s^{-1}) for 7 d, or (B) supplemented with 2% Suc and 0.8% agar (pH 5.8) with (dark gray bars) or without (light gray bars) 5 μM NF under the following conditions: an initial 2 h WL treatment (120 μmol m^{-2} s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 μmol m^{-2} s^{-1}). For A and B, *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to wild-type −NF and normalized to *ACTIN DEPOLYMERIZING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means ±SEM of three independent biological replicates. Asterisks denote a significant difference versus wild type for the same treatment (−NF or +NF), Student's *t* test ($P < 0.05$).

experiment under identical conditions (2% Suc, 4 d dark followed by 3 d 120 μmol m^{-2} s^{-1} WL) to those reported in Sun et al. (2011). Under these conditions we also saw rescue of gene expression in *gun5* and *gun6*, but not in *ptm-1* (Fig. 1B). These studies were performed using *ADF2* as a reference gene. To confirm that the lack of a *gun* phenotype in *ptm1* was not related to the choice of reference gene, we also normalized the data using *YLS8*, which gave essentially identical results (Supplemental Fig. S2). Finally, we examined expression under conditions we have previously described (McCormac and Terry, 2004). With 3 d dark followed by 3 d 120 μmol m^{-2} s^{-1} WL, we also saw no *gun* phenotype for *ptm-1* either in the presence or absence of Suc (Supplemental Fig. S3). Only under one particular set of conditions did we see any indication of a rescue of gene expression in *ptm-1* after NF treatment. Under these conditions (1% Suc, 2 d dark followed by 3 d 100 μmol m^{-2} s^{-1} WL with a lower NF concentration of 1 μM), we saw a very small, but statistically significant increase for *LHCB2.1* and *HEMA1*, but not for the other three genes tested (Supplemental Fig. S4). Given that under these conditions *gun1-1* rescue was complete for both genes ($>$ 300% for *HEMA1*), we do not believe this one exception supports a role for PTM in the plastid signaling pathway exposed by NF treatment.

The *ptm-1* mutant was also reported to result in elevated gene expression compared to wild-type seedlings when grown on Lin (Sun et al., 2011). We therefore also tested *ptm-1* under these conditions. As shown in Figure 2, *ptm-1* failed to result in elevated gene expression on Lin while *gun1-1* (Koussevitzky et al., 2007) and *gun1-103* (see methods) control seedlings both showed strong rescue of gene expression (Fig. 2). This was true whether seedlings were grown in the dark (Fig. 2A) or in the light (Fig. 2B) and was independent of the reference gene used (Supplemental Fig. S5).

To verify further whether we could detect a *gun* mutant phenotype for *ptm* mutants, we also performed experiments in parallel in Kyoto. For this set of

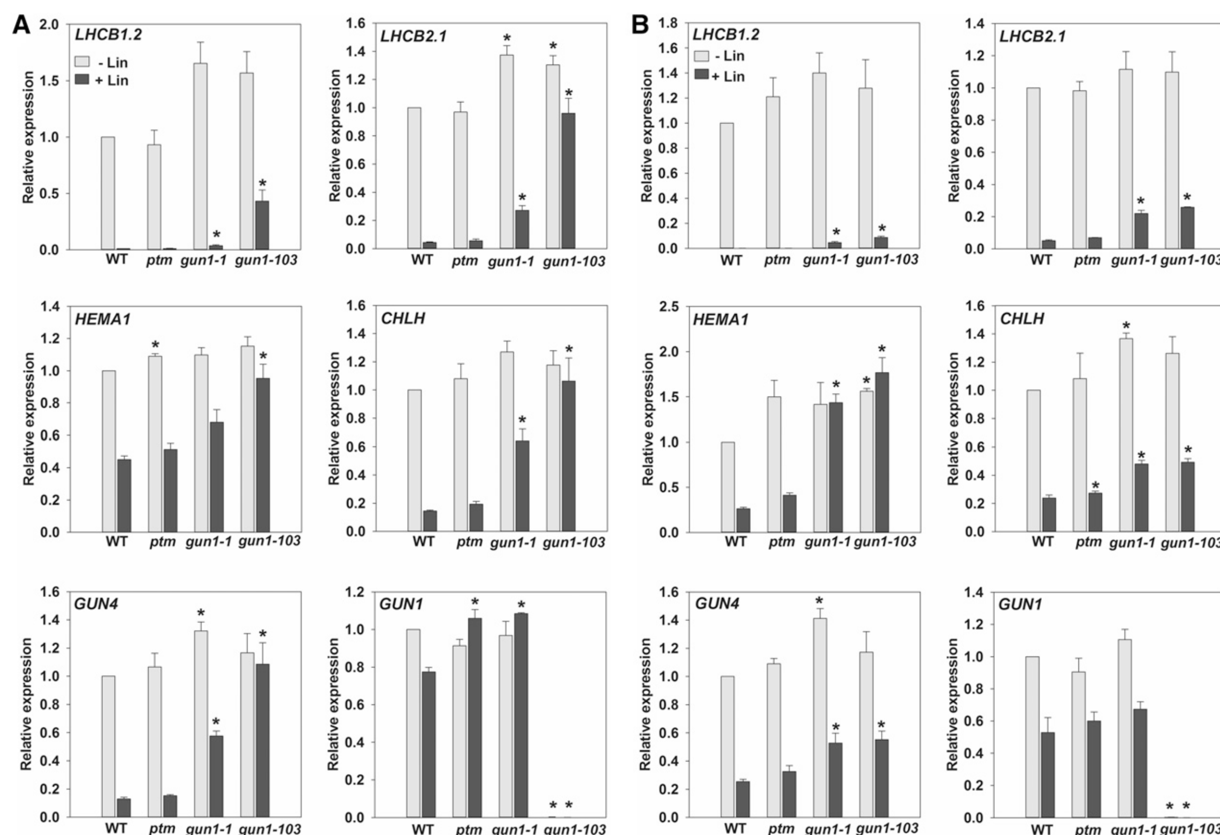

Figure 2. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% Suc and 0.8% agar (pH 5.8) with (dark gray bars) or without (light gray bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% Suc and 1% agar (pH 5.8) with (dark gray bars) or without (light gray bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d WL (100 μmol m^{-2} s^{-1}). For A and B, the *genomes uncoupled, gun1-1*, and *gun1-103* mutants were included as positive controls (known to rescue gene expression on Lin). Expression is relative to wild-type −Lin and normalized to *ACTIN2* (*ACT2*, At3g18780). Data shown are means +SEM of three independent biological replicates. Asterisks denote a significant difference versus wild type for the same treatment (−Lin or + Lin), Student's *t* test ($P < 0.05$).

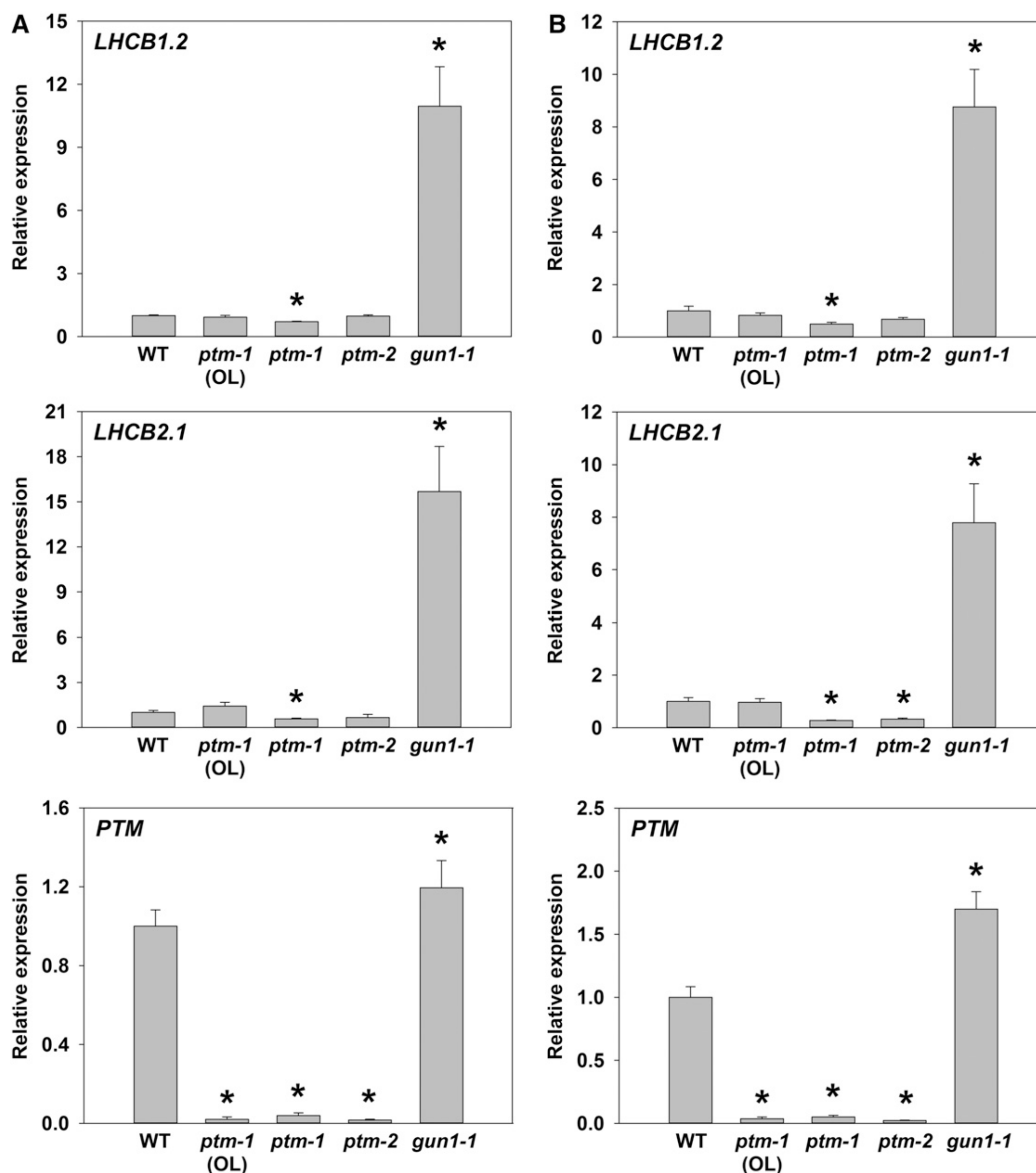

Figure 3. A second *ptm* mutant allele does not show a *gun* phenotype on Norflurazon (NF) or lincomycin (Lin). Seedlings were grown on Murashige and Skoog medium supplemented with 2% Suc and 0.8% agar (pH 5.8), and either (A) 2.5 μM NF or (B) 560 μM Lin. All seedlings were grown under continuous white light (WLc, 100 μmol m^{-2} s^{-1}) for 4 d at 23°C. Three *ptm* mutant lines were tested: *ptm-1* (OL) is the original line as used in Sun et al., 2011; *ptm-1* is the same insertion line as *ptm-1* (OL), Salk_013123, but obtained independently from the stock center; *ptm-2* is a second insertion line, Salk_073799. The *genomes uncoupled 1-1* (*gun1-1*) mutant was included as a positive control (known to rescue nuclear gene expression on NF and Lin). Expression was determined with qRT-PCR and is relative to wild-type +NF (A) or +LIN (B), respectively, and normalized to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the means ±SEM of five independent biological replicates. Asterisks denote a significant difference versus wild-type +NF (A) or +LIN (B), respectively, Student's *t* test ($P < 0.05$).

experiments, two *ptm* alleles were used. The original *ptm* mutant (*ptm-1* OL) was obtained from Lixin Zhang (CAS, Beijing; Sun et al., 2011) and independently from the SALK collection (*ptm-1*) and, in addition, a second *ptm* allele, *ptm-2*, was also identified from the SALK collection (Supplemental Fig. S1). As shown in Figure 3, none of the *ptm* mutants showed an elevation of *LHCB1.2* (although the primer set used is also likely to detect *LHCB1.1* and *LHCB1.3*) or *LHCB2.1* expression after NF or Lin treatment compared to wild type, while a strong increase was observed in the *gun1-1* control.

In conclusion, rigorous testing of the phenotype of *ptm* mutants on NF and Lin shows that the *ptm* mutant does not show elevated expression of photosynthetic

genes compared to wild type. This was true whether using the conditions described in the original publication or other conditions used routinely to test plastid signaling responses. One possible difference between our study and that of Sun et al. (2011) is that they used RNA gel blot analysis for most of their experiments. The probe used should preferentially detect *LHCB1.1*, but might also be expected to detect *LHCB1.2* and *LHCB1.3*, and possibly other *LHCB* genes. In our experiments, we have tested both *LHCB1.1* and *LHCB1.2*, so it remains possible that changes in another *LHCB* gene could account for the observed phenotype in the original paper (Sun et al., 2011). However, Sun et al. (2011) also reported the same gene expression phenotype for *ptm* using real-time PCR and a primer pair that most closely matches *LHCB2.1*, and we did not detect an increase in expression for this gene in our experiments (with one exception). We therefore believe it is unlikely that differences in detection methods or genes tested can account for the observed differences in phenotype. Moreover, if PTM is to be considered an important player in plastid signaling, the *gun* phenotype of *ptm* should be robust enough to withstand this level of scrutiny. We have not tested other results reported by Sun et al. (2011). However, we note that the 3-fold elevation of expression of *PTM* on NF measured using *PTM:GUS* was not apparent in our experiments (Fig. 1 and Supplemental Fig. S3) and the reduction in *PTM* expression in *gun1* after NF and Lin treatment was also not observed (Fig. 3). In fact, *PTM* expression was moderately (but significantly) elevated in *gun1-1* in our study (Fig. 3). Whether our result has implications for other PTM signaling roles (Feng et al., 2016; Xu et al., 2016) is currently unknown, but should be the subject of further scrutiny.

The signaling pathway by which the status of the developing chloroplast is relayed to the nucleus is one of the few remaining plant signaling pathways that we know of, but for which we have little idea of the signaling components involved. We believe this study resolves one of the major discrepancies in plastid signaling research by eliminating a major role for PTM, and paves the way for more focused studies that build on recent progress on the role of tetrapyrroles and chloroplast protein homeostasis in plastid retrograde signaling (Woodson et al., 2011; Maruta et al., 2015; Ibata et al., 2016; Tadini et al., 2016).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Primers used in this study.

Supplemental Figure S1. Characterization of the *ptm* T-DNA insertion mutants

Supplemental Figure S2. The phenotype of *ptm-1* after NF treatment using the Sun et al. (2011) method normalized to *YLS8*

Supplemental Figure S3. The phenotype of *ptm-1* after NF treatment using the McCormac & Terry (2004) method in the presence and absence of Suc

Supplemental Figure S4. The phenotype of *ptm-1* after NF treatment using a modification of the McCormac & Terry (2004) method in the presence of Suc

Supplemental Figure S5. The phenotype of *ptm-1* after Lin treatment normalized to *YLS8*

Supplemental Methods. Supplemental materials and methods.

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