The Arabidopsis SAL1-PAP Pathway: A Case Study for Integrating Chloroplast Retrograde, Light and Hormonal Signaling in Modulating Plant Growth and Development?

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Plants are autotrophs that capture light energy from the sun via photosynthesis. The quantity (intensity) and quality (wavelength) of light can affect the assembly and efficiency of photosynthetic machineries in the chloroplasts (Dietz, 2015), which in turn will determine the amount of carbon available for plants to convert into energy for growth and survival. When optimizing and fine-tuning resource allocation for photosynthesis, plants have to account for both the efficiency of light-harvesting through to electron transport chain under limiting conditions such as low light intensities, and the risk of photodamage by reactive oxygen species (ROS) when conditions flip to the other extremes of excessive light exposure and drought. As such, it has been proposed that the
chloroplast can act as an environmental sensor that subsequently triggers downstream mechanisms for adjustment of plant function and growth according to the environment. Hence, constant communication in the form of anterograde (nucleus to organelles) and retrograde (organelles to nucleus) signaling takes place between chloroplasts and the nucleus to coordinate and maintain cellular function (Chan et al., 2016b).

Leaf/rosette growth and development appears closely associated with chloroplast development and signaling, as well as with the quantity and quality of light and hormonal levels. Phototropism is a classic example of this, which requires mature chloroplasts (Jin et al., 2001), light signaling and hormonal regulation to take place (Fankhauser and Christie, 2015). There is emerging evidence for developmental abnormalities resulting from changes in chloroplast development (Larkin, 2014; Pogson et al., 2015) and from the presence of cells with non-detectable plastids (Chen et al., 2009). Chloroplasts derived from undeveloped proplastids can propagate via division in a light-responsive manner (Hashimoto and Possingham, 1989; Pogson et al., 2015). Both chloroplast development and plant growth are light-dependent, involving phytochrome-mediated signaling (Okello et al., 2016); and all of these are respectively linked to growth hormonal signaling (Halliday and Fankhauser, 2003; Lau and Deng, 2010; Jiang et al., 2012; Yoshizawa et al., 2014). The key growth-regulating hormones in plants are gibberellic acids (GA), brassinosteroids (BR), and auxin; and all of these are respectively involved in the modulating plant growth (de Lucas et al., 2011; Estavillo et al., 2011). Multiple independent studies on PAP-accumulating sal1 mutants suggest additional roles for this metabolite and its catabolic enzyme.

**LOSS-OF-FUNCTION MUTATIONS IN Arabidopsis sal1 GIVE RISE TO DIVERSE PHENOTYPES**

Altered phenotypes related to stress (Lee et al., 1999; Xiong et al., 2001, 2004; Wilson et al., 2009), RNA metabolism (Yu et al., 2007), nutrient uptake (Hirsch et al., 2011), leaf morphology (Robles et al., 2010) or plant hormones (Rodríguez et al., 2010; Zhang et al., 2011) have been associated with mutations in sal1, also known by a diversity of gene names arising from the different alleles characterized (Supplementary Table S1) – hos2 (high expression of osmotically responsive genes), fry1 (firy 1), alx8 (altered expression of APX2 8), ron1 (rotunda 1), fou8 (fatty acid oxygenation up-regulated), and supo1 (suppressor of PIN1 gene expression phenotype). Despite the different ecotype backgrounds such as Col-0 (fry1-4, -5, -6, alx8, fou8, supo1) (Rossel et al., 2006; Yu et al., 2007; Wilson et al., 2009; Rodriguez et al., 2010; Zhang et al., 2011), Ler (ron1) (Robles et al., 2010), C24 (hos2, fry1-1, -2, -3) (Lee et al., 1999; Xiong et al., 2001, 2004), and Ws (fry1-7) (Hirsch et al., 2011), all sal1 knockout mutants consistently display the following developmental phenotypes: delayed development and flowering, more compact rosette with shorter petiole length and rounder leaf shape. Additionally, sal1 was observed to have thicker leaves (Wilson et al., 2009), open venation patterning on leaves and compromised apical dominance based on its increased number of secondary inflorescences compared to wild type (Robles et al., 2010). Altered root morphology was also observed in sal1 mutants (Robles et al., 2010; Hirsch et al., 2011; Zhang et al., 2011). These growth defects coincide with SAL1 expression being detected in all cell types of wild type Arabidopsis throughout development, with higher expression in vascular or vein tissue of leaves and stamens of flowers (Xiong et al., 2001).

Some sal1 growth phenotypes are related to light responses. Kim and von Arnim (2009) showed that sal1 hypocotyl elongation responded similarly to wild type under white light, but was hypersensitive to low-intensity red light, and to a lesser extent, far red and blue light respectively. Crossing a light perception mutant, phytochrome b (phyb), which has much longer petioles, with the sal1 mutant yielded a partial reversion of the altered sal1 rosette morphology. Additionally, Chen and Xiong (2011) demonstrated that an additional long hypocotyl 5
(hy5) mutation in the sal1 mutant can suppress the enhanced light sensitivity of sal1 hypocotyl elongation, but not its altered rosette phenotype. This suggests that sal1 may indeed exhibit altered light perception, signaling or response. As circadian regulation is closely regulated by light signaling (Millar, 2004; Dalchau et al., 2010; Bordage et al., 2016), the effect of PAP accumulation on slightly delaying circadian period (Litthauer et al., 2018) provides further evidence for potential crosstalk between SAL1/PAP and light signaling, although the mechanism remains unclear. Interestingly, a decrease in transitory starch granules was detected in sal1 chloroplasts (Wilson et al., 2009); whether this could be due to this recently reported circadian phenotype of sal1 deserves further investigation since starch metabolism is also closely associated to light and circadian regulation (Webb and Satake, 2015).

The discovery of PAP as a chloroplast stress retrograde signal was initiated when a sal1 knockout mutant axl8 was identified as having constitutive up-regulated expression of ascorbate peroxidase 2 (APX2) – a chloroplast stress marker gene (Rossel et al., 2006; Wilson et al., 2009; Estavillo et al., 2011). Soil-grown sal1 plants can survive 50% longer than wild type during a terminal drought by retaining water more efficiently and maintaining photosynthetic activity (Rossel et al., 2006; Wilson et al., 2009). Consistent with its improved drought tolerance, sal1 has lower stomatal conductance (Rossel et al., 2006; Pornsiriwong et al., 2017), higher accumulation of various osmoprotectants such as putrescine (Wilson et al., 2009), antioxidants and ROS detoxifying enzymes (Rodríguez et al., 2010), jasmonic acid (JA) (Rodríguez et al., 2010) and an inconsistent increase in abscisic acid (ABA) (Rossel et al., 2006; Pornsiriwong et al., 2017). The stress- and ABA-related phenotypes of sal1 were initially hypothesized to be due to inositol polyphosphates (Xiong et al., 2001), which are candidate secondary substrates of SAL1 in vitro (Quintero et al., 1996). This initial hypothesis has been discounted by multiple independent studies that have shown the primary in vivo substrate of SAL1 is PAP, with any change in inositols likely to be indirect secondary effects of SAL1 inactivation (Kim and von Arnim, 2009; Rodríguez et al., 2010; Chen et al., 2011; Estavillo et al., 2011; Lee et al., 2012; Litthauer et al., 2018).

The SAL1-PAP retrograde pathway has been shown to interact with the ABA signaling pathway. Pornsiriwong et al. (2017) demonstrated that PAP-XRN signaling restores ABA-responsiveness of ABA-insensitive mutants [ABA insensitive 1 (abi1), open stomata 1 (ost1)] for stomatal closure; and genetic and exogenous manipulation of PAP increase ABA-responsiveness of seed germination in Arabidopsis. Meanwhile, Chen et al. (2011) observed that the up-regulated ABA signaling in sal1 requires functional ABA Hypersensitive 1 (ABH1), an mRNAs cap binding protein that is involved in ABA signaling. These are in line with Wawer et al. (2018)’s observation that both mRNA decapping and 5’–3’ decay contribute to the regulation of ABA signaling. It will be interesting to further dissect the details of how SAL1-PAP intersects with ABA signaling pathway via its impact on RNA metabolism and whether or not other RNA-independent signaling components are involved.

**COMPLEX INTERACTIONS BETWEEN SAL1-PAP, PLANT HORMONES AND GROWTH**

Evidence presented thus far indicate that the SAL1-PAP chloroplast retrograde pathway crosstalks with light signaling for growth regulation and ABA signaling for stress response regulation. There are other biochemical and physiological phenotypes of sal1: earlier onset of increase in JA levels during vegetative growth (Rodríguez et al., 2010), altered auxin perception/response (Robles et al., 2010), reduced sensitivity toward ethylene (Chen and Xiong, 2010), altered sulfur metabolism (Lee et al., 2012) and enhanced susceptibility toward pathogen attacks (Bruggeman et al., 2016; Ishiga et al., 2017). Evidently, multiple hormonal homeostasis and/or signaling are altered in sal1 and it is common for hormones to crosstalk with one another in coordinating plant growth and stress responses (Jaillais and Chory, 2010; Depuydt and Hardtke, 2011). Therefore, we hypothesized that SAL1-PAP retrograde pathway could also interact with growth hormonal signaling pathway for modulating plant growth.

We investigated whether sal1 growth phenotypes could be an output of deficiency or altered perception/signaling in growth-promoting hormones. For example, the sal1 mutant rosette morphology resembles that of BR-insensitive or -deficient mutants (Caño-Delgado et al., 2004; Rodríguez et al., 2010) and sal1 leaf venation patterning and lateral root formation shares similarities to mutants with altered auxin homeostasis (Robles et al., 2010). Indeed, BR-up-regulated genes are down-regulated in sal1 and vice versa (Robles et al., 2010). Therefore, we supplemented soil-grown sal1 mutant (fry1-6) with the three growth promoting hormones – GA3, auxin [indole-3-acetic acid (IAA)] and epi-brassinolide (EBR) – individually and in combination (at least 10 biological replicates per treatment) from 2 weeks old onwards under 16 h photoperiod, as per method described (Ribeiro et al., 2012). We found that sal1 rosette growth improved the most under the triple hormone treatment, resulting in the largest rosette area after 3 weeks of growth relative to the blank treatment control, followed by GA3 and EBR treatments (Figure 1). Importantly, the response to the hormonal treatments was different in sal1; with sal1 growth complemented best by the triple hormone treatment compared to GA3 alone for wild type (seven biological replicates per treatment) (Figure 1 and Supplementary Figure S1). Furthermore, none of the treatments tested here completely rescued sal1 growth phenotypes.

Partial phenotypic reversion of sal1 was also achieved by crossing the sal1 mutant with the allene oxide synthase (aos) mutant, which has impaired JA biosynthesis and accumulation (Rodríguez et al., 2010). The resulting double mutants have rosette morphology that is partially reverted to wild type-like, specifically the rosette is less compact and has less anthocyanin accumulation but remain smaller than wild type. Hence, part of the altered rosette morphology of sal1 is contributed by the over-accumulation of JA although Ishiga et al. (2017) reported impaired JA signaling in addition to lower levels of SA in sal1 seedlings that correlate with increased its susceptibility to pathogenic attack.
When we analyzed two independent published microarray data of sal1 in Col-0 ecotype background [alx8] (Wilson et al., 2009; Estavillo et al., 2011), we found at least 1500 genes commonly mis-regulated in the same direction (~960 up-regulated, ~550 down-regulated) despite some slight differences in plant age (juvenile-adult vs. adult-flowering stages) and photoperiod (12 vs. 16 h light) (Supplementary Table S2). GO enrichment analyses using DAVID Functional Annotation Clustering revealed that genes involved in translation, defense response, ADP binding and tryptophan biosynthetic processes are significantly enriched in alx8 transcriptomes (Supplementary Table S3), which correlates with the pleiotropic sal1 phenotypes discussed earlier. A substantial number of different hormonal biosynthetic and degradation/inactivation genes (as annotated in the AraCyc/Plant Cyc database) are mis-regulated in the alx8 microarray data. These include up-regulation of genes involved in biosynthesis of ABA, auxin, JA, and GA (CYP88A3 – ent-kaurenoc acid hydroxylase 1); while one BR-inactivation gene and a key chloroplast-localized GA biosynthetic gene GA Requiring 2 (GA2; also known as ent-kaurene synthase) are down-regulated (Supplementary Table S4). Since the GA biosynthetic gene CYP88A3 that is up-regulated in alx8 is positioned downstream of GA2 in the GA biosynthetic pathway, the up-regulation of CYP88A3 is likely a feedback response to the reduced substrate availability.

In order to verify if these transcriptomic alterations impacted the sal1 hormonal profile, we performed quantifications of six different hormones (ABA, JA, auxin, GA, cytokinin, and SA) from leaves of two sal1 mutant alleles (alx8 and fry1-6) in Col-0 (n = 3 to 5 biological replicates) as per method described in (Kanno et al., 2010) (Table 1). Our data revealed for the first time that the sal1 mutants have significantly higher auxin (IAA) and lower GA (GA4) levels

### TABLE 1 | Hormonal quantification results in fold-change for sal1 mutants in comparison to Col-0 wild type at 4 weeks old.

| Hormones          | Col-0 | alx8   | fry-1-6 |
|-------------------|-------|--------|---------|
| Salicylic acid    | 1.06 ± 0.57 | 1.70 ± 0.57 | 1.41 ± 0.26 |
| JA-isoleucine     | 0.99 ± 0.30 | 4.55 ± 5.17 | 3.96 ± 2.14 |
| Jasmonic acid (JA)| 0.96 ± 0.36 | 11.87 ± 10.36 | 7.98 ± 6.08 |
| Indole acetic acid| 1.05 ± 0.25 | 3.67 ± 0.91** | 5.00 ± 1.47** |
| Abscisic acid     | 0.97 ± 0.08 | 1.88 ± 0.37** | 1.73 ± 0.43** |
| Gibberelin (GA4)  | 0.95 ± 0.11 | 0.40 ± 0.07** | 0.48 ± 0.09** |
| t-Zeatin          | 0.99 ± 0.16 | 0.92 ± 0.07 | 0.84 ± 0.20 |
| 2-Isoptenyladenine| 0.96 ± 0.10 | 0.86 ± 0.29 | 0.83 ± 0.39 |

Hormonal profiling was performed on rosette tissues as per methods detailed in Kanno et al. (2010). Means of fold-change ± standard deviation were reported. n = 3 to 5. Significant difference between mutant and wild type was tested based on ANOVA – Dunnett test, where **p-value < 0.01.
relative to the Col-0 control, which correlates with the transcriptomic data. Additionally, we observed higher means and substantive increases in the standard deviations relative to mean for the stress hormones (JA-Ile, JA, and ABA), to an extent that renders JA and JA-Ile levels in sal1 to be not statistically different compared to wild type. Variable increases in ABA and JA have already been reported (Rossel et al., 2006; Rodríguez et al., 2010; Ishiga et al., 2017; Pornsiriwong et al., 2017) and this variability would suggest indirect genotype/development/environmental influences on these hormonal content more than direct PAP signaling regulation of biosynthesis. In this context it is relevant that the stomatal regulation by PAP is primarily due to changes in ABA signaling, not ABA levels (Pornsiriwong et al., 2017). In contrast, there was no change in the content for SA and cytokinins (t-zeatin and isopentenyladenine) in sal1 lines compared to that of wild type (Table 1). Collectively, our hormonal profiling data indicate that sal1 has altered hormonal homeostasis not only in an abiotic stress hormone (ABA) but also in growth regulating hormones (auxin and GA) during the juvenile-to-adult stage of development, which are consistent with the sal1 transcriptome (Supplementary Table S4) and growth complementation by GA and triple hormone treatment (Figure 1).

When considering our hormonal quantification data together with published values it is likely that the effect of PAP accumulation on hormonal homeostasis could be developmental-stage dependent. In contrast to our IAA quantification at the adult stage, Chen and Xiong (2010) did not report differences in auxin levels between the sal1 mutant and wild type at a more juvenile stage. However, sal1 mutant has altered auxin perception (Robles et al., 2010), which could contribute to the increased IAA levels quantified at a later developmental stage, assuming factors like ecotype and growth conditions are negligible. Similarly, no significant changes in SA levels were detected in our experiments in contrast to lower SA levels reported by Ishiga et al. (2017) in sal1 2-week-old seedlings. Since quantification of JA precursor production revealed that the extent of metabolic rate alteration in sal1 differs between developmental stages (Rodriguez et al., 2010) – similar to wild type early on but increase later on – the disparities between the hormonal levels quantified could be due to the different developmental stages of sal1 characterization. Whether or not the increased JA levels at later developmental stages contribute to a readjustment in SA levels in sal1 require further investigation. Therefore, a more systematic and comprehensive profiling of hormones and adenosines throughout sal1 and wild type developmental stages is necessary.

**HUNTING FOR THE MEDIATORS OF SAL1-PAP, LIGHT AND HORMONAL REGULATION OF PLANT GROWTH**

What are the possible intersection points between SAL1-PAP chloroplast signaling, hormonal and light signaling? The signaling network is likely to be complex and comprise several components which have been demonstrated to contribute to sal1 phenotypes; and participate in both hormonal and light signaling. In our view, tempting candidates would include, but not be limited to, the aforementioned RNA metabolism, PHYB, HY5, as well as DELLA and BR signaling proteins.

Our observation of lower GA levels in sal1 could suggest for more abundant DELLA proteins in sal1 compared to wild type since GA promotes DELLA degradation. DELLA proteins can physically interact with key hormone-responsive transcription factors such as JA ZIP-domain family proteins (JAZs) (Hou et al., 2010) and brassinazole resistant 1 (BZR1) (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012) to promote GA signaling, which can feedback into promoting DELLA expression (Wild et al., 2012) and repress BR signaling (Supplementary Figure S2). Intriguingly, DELLA proteins also mediate light responses via their interactions with phytochrome-interacting factors (PIFs) proteins (de Lucas et al., 2008; Feng et al., 2008). Furthermore, constitutive higher DELLA accumulation as a result of low GA levels can activate ABA biosynthesis (Piskurewicz et al., 2008) and confer drought tolerance to Arabidopsis (Colebrook et al., 2014). These features correlate with multiple reported growth and hormonal-related phenotypes of sal1 discussed above, and it is interesting to note that transcript levels of a few DELLA proteins are up-regulated in ak8 (Estavillo et al., 2011). However, whether this transcriptional up-regulation genuinely reflects increased protein abundance is unclear and detailed work is required to mechanistically dissect how PAP and GA together with other hormonal signaling are interlinked.

It will also be critical to further unravel the interaction between PAP and the secondary messengers ROS and Ca$^{2+}$. Both of these messengers are known to be key components of hormonal signaling. They are also involved in light signaling and can crosstalk with one another for cellular signaling (Mazars et al., 2010; Gilroy et al., 2014; Gőrlach et al., 2015). It is intriguing that PAP has contrasting effects on ROS depending on cell and tissue type; inducing a ROS burst in guard cells associated with the restoration of stomatal closure in ABA insensitive mutants (Pornsiriwong et al., 2017) while suppressing ROS levels in vascular tissue (Wilson et al., 2009). Similarly, increased Ca$^{2+}$ levels in roots was reported in one of the mutant alleles of sal1 (Zhang et al., 2011), but exogenous PAP treatment does not induce any significant changes in cytosolic Ca$^{2+}$ transients in guard cells (Pornsiriwong et al., 2017). Intracellular ROS and Ca$^{2+}$ homeostasis are known to regulate cell growth in leaves and germinating seed (Conn et al., 2011; Leymarie et al., 2012); though the exact mechanisms are still unclear. It will be interesting to explore whether and how PAP can contribute to these processes.

**CONCLUDING REMARKS**

The numerous studies on sal1 to date reveal that the SAL1-PAP chloroplast retrograde signaling pathway in Arabidopsis can
intersect with light signaling and hormonal signaling pathway in affecting, if not regulating, plant growth and development. While chloroplast retrograde, light and hormonal signaling pathways have been studied independently, and to some extent in a two-way crosstalk manner in the past; the SAL1-PAP pathway may provide the platform to investigate crosstalk between the three pathways altogether in regulating plant growth and development in response to environmental changes (i.e., sun vs. shade) at the molecular level.

**AUTHOR CONTRIBUTIONS**

SYP performed the transcriptomic mining and hormonal treatment experiment. DY and EN performed the hormonal quantification of sall. SYP, KXC, GME, and BJP led planning, analysis, and manuscript preparation.

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**SUPPLEMENTARY MATERIAL**

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Phua et al.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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