Plasmodesmal closure elicits stress responses

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Abstract

Plant cells are connected to their neighbors via plasmodesmata facilitating the exchange of nutrients, hormones, and signaling molecules. While immune elicitors trigger changes in plasmodesmal aperture, it is unknown how this contributes towards a full immune response. To investigate this, we developed two transgenic lines with which we could induce plasmodesmal closure independently of immune elicitors. Thus, we used the over-active CALLOSE SYNTHASE3 allele *icals3m* and the C-terminus of PDLP1 to drive callose deposition at plasmodesmata in response to estradiol treatment. These lines showed different degrees of plasmodesmal closure but both showed upregulation of the expression of stress responsive genes, the accumulation of salicylic acid and enhanced resistance to *Pseudomonas syringae* DC3000 in response to estradiol treatment. Enhanced resistance was not correlated with perturbation of the flg22-triggered ROS burst or MAPK signaling. Enhanced plasmodesmal closure using icals3m also led to the measurable accumulation of starch and sugars, decreased leaf growth, and induced leaf senescence. Additionally, induction of plasmodesmal closure using icals3m led to hypersusceptibility to *Botrytis cinerea*. We conclude that plasmodesmal closure itself can activate stress signaling, raising questions of how this occurs, what signal carries this information and whether these responses occur in all circumstances when plasmodesmata close across plant physiology.

Introduction

Plants are multicellular organisms, with almost every plant cell cytoplasmically connected to its neighbors. While most plant cells are equipped with machinery to respond autonomously to a range of stress signals, optimal responses involve the regulation of this cytoplasmic connectivity between cells. This is particularly evident in immune signaling; most cells produce the receptors that perceive microbial threats and can activate appropriate responses. However, cells execute these while connected to their neighbors and regulate their degree of connectivity to neighboring cells as part of the full immune response. If plants cannot regulate their cytoplasmic connectivity between cells they are more susceptible to infection by a range of pathogenic microbes, but how cell-to-cell connectivity contributes to an overall immune response is an outstanding question.

Cytoplasmic connectivity between plant cells is established via membrane-lined bridges called plasmodesmata. Plasmodesmata are dynamic structures and the oscillation in their aperture, i.e., closing and opening, allows control over the flux of soluble molecules between cells. We broadly assume that many molecules that move through plasmodesmata, including plant hormones, mRNA, and proteins, are information carriers; therefore, aperture changes impact cell-to-cell communication. Indeed, if an immune response involves the production of soluble defense-associated molecules, their passage through plasmodesmata could transmit critical information to neighboring (or distant) naïve cells.

Plant immune responses include an upregulation of callose synthesis at plasmodesmata leading to a decrease in plasmodesmal aperture, which reduces cell-to-cell movement of molecules (as recently reviewed by Wang et al. 2021b; German et al. 2023; Alazem and Burch-Smith 2024). The plasmodesmal aperture itself is regulated via callose deposition and degradation at the plasmodesmal neck by callose synthases (CalS) and β-glucanases respectively (Levy et al. 2007; Vatén et al. 2011). The decrease in plasmodesmal aperture in response to stress is a process activated by signaling cascades mediated by specific machinery. For example, LYSM-CONTAINING GPI-ANCHORED PROTEIN 2 (LYM2; Faulkner et al. 2013) and CALMODULIN-LIKE 41 (CML41; Xu et al. 2017) specifically mediate plasmodesmal responses to the microbial elicitors chitin and flg22 respectively, and

PLASMODESMATA LOCATED PROTEIN 5 (PDLP5) integrates these responses, as well as plasmodesmal responses triggered by the defense hormone salicylic acid (SA; Wang et al. 2013; Tee et al. 2023b). The observation that *lym2* mutants cannot close their plasmodesmata in response to chitin but execute normal chitin-triggered mitogen-activated protein kinase (MAPK) activation and apoplastic ROS production (Faulkner et al. 2013) indicates that plasmodesmal signaling cascades can act independently of other responses. This independence of response suggests that whether plasmodesmata are open or closed might be informed by another layer of cellular regulation.

Whether plasmodesmata can close or not during immune responses determines whether a full defense response can be executed, but untangling how plasmodesmal closure contributes to overall immunity is a complex problem. To begin to address this we have simplified the question and asked what cellular responses are triggered by plasmodesmal closure, and how closing plasmodesmata affects different immune responses. We generated tools with which we can induce plasmodesmal closure independently of an immune or stress elicitor and found that plasmodesmal closure itself instigates a subset of stress responses including transcriptional reprogramming and SA production. This raises further questions regarding how this contributes to the orchestration of immune responses, how plasmodesmal closure triggers stress responses and whether these stress responses occur during the broad range of physiological processes during which plasmodesmata close.

Results

Genetic tools induce plasmodesmal closure independent of a physiological elicitor

To manipulate plasmodesmal closure independently of external signals we generated two transgenic lines in which estradiol application can induce callose deposition at plasmodesmata. First, we exploited the *icals3m* overactive *CalS3* allele (Vatén et al. 2011) that enhances callose deposition at plasmodesmata. Secondly, we utilized the observation that a synthetic peptide that fuses the PDLP1 signal peptide to the 5’ end of mCherry and the PDLP1 transmembrane domain and cytoplasmic tail to the 3’ end also promotes callose deposition at plasmodesmata (Caillaud et al. 2014). We fused these independently to the *XVE* chimeric transcription activator and *LexA* operator

(Zuo et al. 2000) and transformed them into *Arabidopsis thaliana* Col-0 to create two different inducible tools, named LexA::icals3m and LexA::PD-Plug respectively. To identify functional transgenic lines, we performed qPCR, copy number analysis and/or live imaging (Supplemental Table 1; Supplemental Fig. S1). To functionally characterize the selected LexA::PD-Plug and LexA::icals3m lines, we first measured callose deposition by quantitative live cell imaging of aniline blue stained plasmodesmal callose. We found that there was an increase in callose deposition at plasmodesmata 24 h post estradiol treatment in both LexA::PD-Plug and LexA::icals3m when compared to the DMSO treatment, although there was a greater response in LexA::icals3m (Fig. 1a; Supplemental Fig. S2) suggesting that this line deposited more callose at plasmodesmata than LexA::PD-Plug. To determine whether increased callose deposition perturbed plasmodesmal permeability, we used microprojectile bombardment assays of *35S::GFP* to measure GFP flux between cells in DMSO or estradiol treated leaves. While estradiol treatment did not impact GFP flux in comparison to DMSO in Col-0 plants, we found that estradiol induction of either transgene reduced GFP flux between cells, indicating plasmodesmal aperture was reduced and cell-to-cell connectivity decreased (Fig. 1b-c; Supplemental Data Set 1). Furthermore, correlated with increased plasmodesmal callose in LexA::icals3m, the extent of GFP flux was significantly decreased in induced LexA::icals3m compared to induced LexA::PD-Plug (Fig. 1c).

From these results, we can conclude that estradiol application to both LexA::PD-Plug and LexA::icals3m, but not Col-0, induces plasmodesmal callose deposition and plasmodesmal closure. Therefore, these transgenic lines allow us to manipulate plasmodesmata independently of both endogenous and non-endogenous signals and investigate plant responses to plasmodesmal closure.

Plasmodesmal closure differentially impacts pathogen resistance and immune responses

Plants that lack plasmodesmata-specific immune signaling machinery cannot close their plasmodesmata in response to pathogen infection and show increased susceptibility (Lee et al. 2011; Faulkner et al. 2013; Xu et al. 2017). Thus, we hypothesized that increased plasmodesmal closure enhances defense. An overall

indication of defense success is pathogen resistance. To test whether induced plasmodesmal closure enhances resistance to pathogens, we examined the susceptibility of induced, transgenic lines to the bacterial, biotrophic pathogen *Pseudomonas syringae* DC3000 (*Pst* DC3000). We inoculated mature leaves of Col-0, LexA::PD-Plug and LexA::icals3m plants 72 h post DMSO or estradiol treatment (Fig. 2a) and found that induction of both transgenes reduced bacterial growth 3 days post infection (dpi). Thus, plasmodesmal closure is correlated with enhanced resistance to *Pst* DC3000. Next, we assayed for resistance to the fungal necrotroph, *Botrytis cinerea*. In contrast with *Pst* DC3000 infection, we found induction of the PD-Plug transgene had no impact on *B. cinerea* infection when compared to the disease caused in Col-0 plants, but instead induction of *icals3m* increased susceptibility to *B. cinerea* as indicated by an increased disease lesion size (Fig. 2b; Supplemental Fig. S3). This indicates that plasmodesmal closure specifically does not enhance resistance in all infection contexts.

Overall susceptibility is driven by multiple components of host defense responses, and plasmodesmal closure is a key immune response to the pathogen associated molecular patterns (PAMPs), flg22 and chitin. Previously, we have observed that PAMP-triggered plasmodesmal closure occurs independently of other PAMP responses (Faulkner et al. 2013; Xu et al. 2017). To confirm that plasmodesmal closure in our transgenic lines is likewise independent of PAMP responses and this is not the cause of the increased resistance we observed to *Pst* DC3000, we examined the effect of plasmodesmal closure on the activation of MAPK signaling and ROS production by flg22. When we assayed for flg22-triggered MAPK phosphorylation in LexA::PD-Plug and LexA::icals3m lines following estradiol treatment, we saw no difference in the timing or magnitude of the response in either line when compared to Col-0 in mature leaves and seedlings (Fig. 2c; Supplemental Fig. S4). However, while induction of *LexA::PD-Plug* did not perturb the flg22-triggered ROS burst in either seedlings or mature leaves (Fig. 2d,e), when we assayed for the same response in LexA::icals3m, induction of the transgene reduced the flg22-triggered ROS burst in mature leaves (Fig. 2d) and increased the response in seedlings (Fig. 2e). To determine whether there were differences in general basal cellular ROS levels that might explain this observation, we stained estradiol treated Col-0 and LexA::icals3m leaves with H_2DCFDA but observed no quantitative differences between the

genotypes or treatments (Supplemental Fig. S5), indicating plasmodesmal closure itself does not alter basal ROS cellular levels.

These results indicate that plasmodesmal closure differentially influences the outcomes of host interactions with different pathogenic microbes, possibly correlated with differences in pathogen lifestyle. Increased resistance to *Pst* DC3000 is not associated with enhanced MAPK activation or, given that our pathoassays were performed on mature leaves, changes in ROS burst responses. Therefore, resistance must arise from other processes that positively regulate defense. Further, our ROS data raises the possibility that plasmodesmal closure differentially interacts with immune responses in different developmental stages.

Plasmodesmal closure triggers stress transcriptional responses and salicylic acid production

To explore what other immune associated processes might underly the positive correlation between *Pst* DC3000 resistance and plasmodesmal closure we transcriptionally profiled responses to plasmodesmal closure using RNAseq. For this, we harvested leaf tissue at 12 h, 24 h, 48 h and 72 h following DMSO or estradiol treatment.

Preliminary analysis of the data first revealed that a critical driving factor of variance was time (Supplemental Fig. S6). Therefore, all subsequent analyses were performed within single time points. We assessed the effect of estradiol relative to DMSO treatment within a genotype, examining differentially expressed genes (DEGs). The greatest response was in Col-0 at 12 h, where estradiol led to the downregulation of over a thousand genes (Supplemental Fig. S7). Shared differentially expressed genes in response to estradiol in Col-0 and the transgenic genotypes imply an estradiol specific response not correlated to transgene induction; these genes were dramatically reduced after 24 h (Supplemental Data Set 2; Supplemental Fig. S7), suggesting secondary effects of estradiol are negligible after this point. Assessing transgene induction across this sampling period, both the PD-Plug transcripts (as indicated by read counts of mCherry) and icals3m transcripts (as indicated by read counts of CalS3) were upregulated compared to controls by 12 h post estradiol treatment and remained significantly elevated at 72 h (Supplemental Fig. S8).

To identify genes which are uniquely upregulated when plasmodesmata are closed, we generated two different groups to draw comparisons: plasmodesmal state closed (estradiol-treated LexA::PD-Plug and LexA::icals3m) and plasmodesmal state open (DMSO treated LexA::PD-Plug and LexA::icals3m, and all Col-0 samples). A likelihood-ratio test was performed on these two groupings at each time point to identify genes that were significantly differentially expressed only in the closed plasmodesmal state. To identify what cellular processes were disrupted or initiated, we further selected significantly upregulated DEGs when plasmodesmata were closed (Fig. 3a; Supplemental Fig. S11; Supplemental Data Set 3-6) and observed that the number of upregulated DEGs increased over time, suggesting that the longer plasmodesmata remain closed, the greater the cellular response. A GO term analysis identified that genes upregulated when plasmodesmata are closed are enriched in defensive processes, specifically at 48 and 72 h (Fig. 3b; Supplemental Fig. S12). These include response to bacterium, oomycetes, chitin and fungus, regulation of systemic acquired resistance, and response to salicylic acid (SA).

As response to SA at 72 h was the most significantly enriched GO term in the entire analysis, we further explored this by analyzing DEGs related to SA to determine what aspects of SA signaling were impacted when plasmodesmata were closed (Fig. 3c; see Supplemental Data Set 7 for associated references; Vlot et al. 2008; Seyfferth et al. 2014; Yang et al. 2015; van Butselaar and Van den Ackerveken 2020) and found both SA biosynthesis/catabolism and SA response pathways were significantly upregulated at 48 and 72 h. To determine whether the transcriptional signature of SA is a consequence of the activation of SA synthesis when plasmodesmata are closed, we measured SA content in Col-0, LexA::PD-Plug and LexA::icalsm3 72 h post DMSO and estradiol treatment. We found induced plasmodesmal closure increased SA content in both LexA::PD-Plug and LexA::icals3m, with induced LexA::icals3m having a greatly increased SA content, being three-fold higher when compared to DMSO treatment (Fig. 3d). Thus plasmodesmal closure leads to SA synthesis and accumulation independently of an immune stimulus.

Plasmodesmal closure induces sugar accumulation and growth defects

Given the increased defense related transcripts, we next explored how these modulated differences in plasmodesmal permeability could translate phenotypically. As plasmodesmata connect the phloem to cells in young sink tissues and allow sugar translocation, we expect that plasmodesmal closure might reduce the flux of sugars to growing tissues and limit growth. Alternatively, activation of defense processes negatively regulate growth and therefore it is possible that plasmodesmal closure impairs growth by one or both pathways. Indeed, several mutants known for constitutive plasmodesmal closure exhibit a significant retardation in growth such as *PDLP1* and *PDLP5* overexpressors (Thomas et al. 2008; Lee et al. 2011). Thus, we explored whether sustained plasmodesmal closure reduced growth in induced LexA::PD-Plug and LexA::icals3m. In 9-day old seedlings, we applied either DMSO or estradiol treatment and measured the first true leaves (Fig. 4a) six days post treatment. We found that estradiol reduced the leaf area in both Col-0 and LexA::PD-Plug in comparison to the DMSO treated seedlings. Further, the estradiol-induced growth reduction was greater in LexA::icals3m relative to LexA::PD-Plug and Col-0. Alongside the reduced leaf size, we observed yellowing and chlorosis on the estradiol treated LexA::icals3m true leaves (Supplemental Fig. S13).

Plasmodesmal closure might be expected to restrict photoassimilate distribution between source and sink tissue, and indeed, a reduction in plasmodesmal permeability and rosette size has been documented in *Arabidopsis* alongside a significant accumulation of soluble sugars and starch (e.g., in the MOVEMENT PROTEIN 17 *MP17* overexpressor and *CHOLINE TRASPORTER-LIKE 1 cher1* mutant; Kronberg et al. 2007; Kraner et al. 2017). Therefore, we quantified sugar accumulation in source tissues following plasmodesmal closure, measuring the sucrose, fructose, glucose, and starch content of source leaves of plants 72 h post treatment. These data reveal a significant increase in all four sugars in estradiol treated LexA::icals3m samples (Fig. 4b). Reasoning that an increase in sugar content might down-regulate photosynthesis, we used chlorophyll fluorescence imaging to infer photosynthetic yield and found there was no difference in photosynthetic yield of PSII (as indicated by Y(II)) after 72 h in Col-0, LexA::PD-Plug and LexA::icals3m between the DMSO or estradiol treatments (Table 1). As there were no changes in the photosynthetic yield that correlated with the increase in sugar content in induced LexA::icals3m, we next hypothesized that plasmodesmal closure and sugar

accumulation might activate the expression of sugar transporters and starch biosynthesis genes to reduce the concentration of soluble sugars in the cytosol. However, out of 125 genes known to be sugar and starch related genes (Supplemental Data Set 8; Büttner and Sauer 2000; Williams et al. 2000; Chen et al. 2010; Monroe and Storm 2018; Abt and Zeeman 2020; Preiser et al. 2020; Smith and Zeeman 2020; David et al. 2022; Bavnhøj et al. 2023), only the transporter *SENESCENCE-ASSOCIATED GENE 29 (SAG29)/SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS 15 (SWEET15)* was significantly upregulated when plasmodesmata are closed, in LexA::icals3m at 48h and 72 h (Supplemental Fig. S14, S15). AT3G20460 was differentially expressed in LexA::icals3m estradiol treatment at 72 h and is proposed to be a monosaccharide transporter (Johnson et al. 2006). However, this gene was downregulated in response to plasmodesmal closure and as the function of this gene is not yet well characterized it is difficult to speculate on any possible role in the sugar/starch phenotype (Johnson et al. 2006). These results indicate that while plasmodesmal closure induces starch and soluble sugar accumulation, it does not perturb photosynthesis or the expression of sugar transporters.

Discussion

Plants initiate a suite of defense responses when challenged by a microbial threat. Plasmodesmal closure is an early reaction, initiated within 30 minutes upon perceiving pathogen-derived molecules and stress-associated signals including SA and hydrogen peroxide (Xu et al. 2017; Cheval et al. 2020; Tee et al. 2023b), and is critical for the execution of a full response. Thus, we reason that plasmodesmal closure regulates a range of downstream processes. However, considering the plethora of responses elicited during immunity, and the array of elicitors that trigger plasmodesmal closure via converging signaling pathways (Tee et al. 2023b), it is challenging to identify what specific contribution plasmodesmal closure makes to the overall execution of immunity. Using lines with which we can induce plasmodesmal closure independently of elicitors, we explored what occurs when plasmodesmata close without other co-incident processes and determined that plasmodesmal closure itself induces stress.

Transcriptional profiling of responses to plasmodesmal closure identified transcriptional signatures of defense responses associated with SA production and signaling. It has long been known that constitutive over-production of the plasmodesmal protein PDLP5 closes plasmodesmata and leads to high levels of SA (Wang et al. 2013). However, as PDLP5 is transcriptionally responsive to SA it is viewed as stress response element (Wang et al. 2013). We have previously shown that microbial elicitors, flg22 and chitin, also require PDLP5 to elicit plasmodesmal closure, but these elicitors interestingly do not require ISOCHORISMATE SYNTHASE 1 which is involved in the synthesis of SA (Wildermuth et al. 2001), for the plasmodesmal response (Tee et al. 2023b). This poses a potential model in which plasmodesmal closure is first initiated upon pathogen perception, leading to the accumulation of SA at plasmodesmata to create a feedback loop to sustain closure. Our finding that plasmodesmal closure leads to SA accumulation also raises the question of whether plasmodesmal closure in all contexts induces SA synthesis and accumulation, and thus if SA plays a role in developmental contexts which require plasmodesmal closure.

The accumulation of SA when plasmodesmata are closed might be elicited directly in response to plasmodesmal signaling, or indirectly as a secondary consequence of another process triggered by a change in cellular status induced by isolation. The greater accumulation of SA observed in induced LexA::icals3m occurs in parallel with an observed increase in sugar levels (Fig. 4c) and is correlated with the greater degree of plasmodesmal closure observed in this line relative to LexA::PD-Plug. It is possible that the increase in sugar content induces osmotic stress or another process that feeds into SA signaling, and therefore that these stress responses are secondary to plasmodesmal closure. Further, the combined increase in sugar content and SA might explain our pathoassay data; the necrotrophic pathogen *B. cinerea* might be able to access increased intracellular sugar stores and be unimpeded by SA defense pathways (Ferrari et al. 2003; El-Oirdi et al. 2011), while *Pst* DC3000 might be unable to access intracellular sugars and is impeded by SA (Velásquez et al. 2017; Wilson et al. 2017; Howlader et al. 2020).

Sugars can induce specific signal transduction pathways, with some studies finding that increasing source leaf carbohydrate content leads to a feedback loop of decreasing photosynthetic yield (Araya et al. 2006). Additionally, an inverse

relationship between plasmodesmal aperture and the expression/activity of sugar transporters (Ruan et al. 2001; Zhang et al. 2017; Liu et al. 2022) has been reported suggesting that cells maintain the capability to transport sugars between cells by balancing plasmodesmal aperture with transporter activity (Zhang et al. 2017). On this basis, we might expect that induction of LexA::icals3m would activate a compensatory mechanism for sugar distribution. However, we only observed that one sugar transporter was upregulated in induced LexA::icals3m, SAG29/SWEET15, which is also activated by SA during the process of leaf senescence (Seo et al. 2011; Wang et al. 2021a). Therefore, while this may be a component of the senescing phenotype observed in induced LexA::icals3m, the increased expression of this transporter does not counteract the intracellular sugar accumulation observed (Chen et al. 2015). Irrespective, the question arises as to whether there is a cellular mechanism that perceives an increase in soluble sugars and whether this is incapacitated when plasmodesmata are closed independently of other signal inputs.

The variance in multiple sets of experimental data suggest that physiological plasmodesmal responses are heterogeneous within a tissue, i.e., not all cells close their plasmodesmata in response to an elicitor such as chitin or flg22 (Faulkner et al. 2013; Xu et al. 2017; Cheval et al. 2020; Tee et al. 2023b). Our transgenic lines show two different patterns of plasmodesmal response: we see variability in GFP mobility in the induced LexA::PD-Plug line that resembles physiological data sets despite homogeneous induction of the transgene (Supplemental Fig. S1), while data from induced LexA::icals3m (Fig. 1b) exhibits much lower variance. This suggests that more cells are isolated from their neighbors than normally occurs and LexA::icals3m induces plasmodesmal closure more homogeneously than in LexA::PD-Plug. This increased homogeneity of plasmodesmal closure correlates with the magnitude of the responses measured in most of the assays performed in this study, with effects on disease resistance, growth, ROS burst, sugar and SA accumulation all greater in magnitude in, or only detected in, LexA::icals3m relative to LexA::PD-Plug. Thus, our data suggests that we have detected some of these effects only when they have been elicited in more cells. However, an alternative possibility is that some responses to plasmodesmal closure occur only when a threshold of isolated cells is reached, which would imply that responses to plasmodesmal closure is a tissue level response, and that connectivity is a population-level parameter.

In this study we have induced plasmodesmal closure in a non-physiological manner to isolate it from other immune responses and study its effects. This raises the consideration of how responses to induced plasmodesmal closure correspond to the effects of plasmodesmal closure that occurs as a physiological response to stress. For example, how long plasmodesmal closure is sustained in different response contexts is not well described, and whether SA synthesis and accumulation occur within these timeframes is an important question. However, during an infection, the exposure of cells to PAMPs is likely to be a sustained process suggesting that prolonged plasmodesmal closure occurs during infection. Further, given we have identified plasmodesmal closure without immune activation triggers stress-associated responses, whether these responses occur when cells are isolated during developmental transitions must also be considered. Likewise, in many investigations into developmental transitions, the icals3m tool is used to induce ectopic plasmodesmal closure at different cell boundaries and our data indicates that this is also likely to block the distribution of sugars to, or from, targeted cells and activate defense. Thus, phenotypes that arise from blocking plasmodesmata at a critical developmental transition must be interpreted with these factors in mind.

We have found that plasmodesmal closure triggers stress responses. This suggests that cellular isolation is a stress-inducing state and, conversely, that connection to neighboring cells is essential for optimal growth-associated cellular functions. Thus, understanding how a cell responds when it becomes isolated from its neighbors, and why cell-to-cell connectivity regulates homeostatic cellular processes, is key to understanding how plants function as multicellular organisms.

Methods

Plant material

All *Arabidopsis thaliana* used in this study is in the Columbia-0 ecotype background. For assays on mature plants, *Arabidopsis* plants were grown at 10 h light at 22 °C, on soil or for the RNA-seq experiment, on Murashige and Skoog (MS) 1% sucrose 0.8% agar in 50 mm diameter petri dishes for 4–5 weeks. For MAPK and ROS measurement assays on seedlings, seeds were germinated on MS 1% sucrose 0.8% agar at 16 h

light at 22 °C and grown for 8 days, then 10 seedlings were transferred per well on a 6-well plate together with 8 mL of liquid MS and grown for an additional 7 days.

Confocal microscopy

Microprojectile bombardment, transgene induction visualization and aniline blue plasmodesmal callose measurements utilized confocal microscopy (LSM Zeiss 800). RFP/mCherry was excited with a 561-nm DPSS laser and collected at 600 to 640 nm, GFP/H_2DCFDA was excited with a 488-nm argon laser and collected at 505 to 530 nm, and aniline blue was excited with a 405-nm UV laser and collected at 430 to 550 nm.

Microprojectile bombardment

Microprojectile bombardment of *35S::GFP* was performed as described in (Tee et al. 2022). 24 h after DMSO or estradiol treatment, 5-week-old leaves were co-bombarded with pB7WG2.0.RFP_{ER} and pB7WG2.0.GFP. Bombardment sites were then imaged 16 h after using confocal microscopy with a 20× water dipping objective (W N-Achroplan 20×/0.5).

Generation of transgenic lines and induction of transgene

The *LexA::icals3m* construct is as described (Bellandi et al. 2022). The *LexA::PD-Plug* construct was assembled into a binary vector via Golden Gate cloning; first the 'PD-Plug' sequence was created by fusing the PDLP1 signal peptide and the PDLP1 transmembrane domain and cytoplasmic tail to the N- and C-termini of mCherry respectively (Caillaud et al. 2014). This synthetic peptide was inserted downstream of the *LexA* promoter sequence and upstream of a NOS terminator and assembled into the backbone of pICSL0022013 containing the *Nos::BAR::Nos* selection cassette and *Act2::XVE::Act2* cassette.

LexA::PD-Plug and *LexA::icals3m* constructs were transformed into the Col-0 *Arabidopsis* background and screened with phosphinothricin (10 µg/mL concentration) or kanamycin selection (50 µg/mL concentration) respectively. For induction of the transgene in all experiments (with the exception of size phenotyping), leaves were painted on both the abaxial and adaxial side with either 0.1% DMSO (mock treatment) or 20 µM β-Estradiol 17-acetate (estradiol treatment) containing 0.01% Silwet L-77 in

ddH_2O, using a fine paint brush. For size phenotyping, seedlings were sprayed with the above specified DMSO or estradiol treatment.

Plasmodesmal callose measurements

24 h post treatment with either DMSO or estradiol, expanded leaves of 5 week old plants were infiltrated with 1% aniline blue in PBS buffer (pH 7.4) then imaged using confocal microscopy from the abaxial side using a 63× water immersion objective (Plan-Apochromat 63×/1.20). Z-stacks from multiple areas from a given treated leaf, with a minimum of three biological replicates per genotype and treatment were collected. An automated image analysis pipeline to quantify aniline blue-stained plasmodesmata is available at (Johnston et al. 2022). Raw image data will be available on the BioImage Archive.

Quantitative PCR

To determine transgene expression, *Arabidopsis* T2 seedlings were grown on MS 1% sucrose 0.8% agar with phosphinothricin (10 µg/mL concentration) or kanamycin (50 µg/mL concentration), for 21 days in 16 h light at 22 °C. Leaves from each line was treated with DMSO or estradiol as above and harvested 48 h later in liquid nitrogen. Samples were homogenized in a GenoGrinder 2100, with RNA extracted, DNase treatment, first strand cDNA synthesis and qPCR performed as previously described (Bellandi et al. 2022). Genes tested are *icals3m*/*CalS3* (using primers AGGACGTTACTTCATGGCGG and GGCTCCGGGAATACAGTCTG) and the housekeeper *AtUBQ10* (using primers AGTCTACTCTTCACTTGGTCCTGC and GCCCCAAAACACAAACCACCAAAG). Normalized relative quantities (NRQs) were generated using the qBase model (Hellemans et al. 2008).

Pseudomonas syringae infection assays

Three leaves from 4–5-week-old plants were treated with DMSO or estradiol. After 72 h, leaves were infiltrated with 5×10^4 cfu/mL *Pseudomonas syringae* DC3000 and harvested 72 h later. Each leaf was harvested with a 5 mm corer and the three leaves from a single plant combined as a sample. Leaf tissue was homogenized with 1 mL ddH_2O for 2 mins at 1200 rpm in a GenoGrinder 2100. 5 µL of multiple 10-fold dilutions were plated out with three technical replicates per plant sample, then incubated for 48

h at 28 °C with colony counts determined from countable dilutions. Four independent experiments with five plants per treatment/genotype per experiment was performed.

Botrytis cinerea infection assays

B. cinerea spores were applied to leaves from 4–5-week-old plants 72 h post DMSO or estradiol treatment. *B. cinerea* spores were harvested and adjusted to a concentration of 2.5×10^5 spores/mL in 0.25× potato dextrose broth and incubated at room temperature for 2 h with continual shaking for spore germination. Leaves were adhered to 1.5% agar H_2O plates, and droplets of 2 µL spore suspensions were placed in between the mid vein and leaf edge on each side. Plates were sealed with parafilm and incubated in a cabinet set at 22 °C with 10 h light. Developing disease lesions were photographed 2 dpi and measured using FIJI. Each prepared plate had four leaves per genotype and treatment, and the average lesion area was calculated per genotype/treatment in a given plate, defined as a single biological replicate. Three independent experiments were conducted with a minimum of five biological replicates per genotype/treatment.

MAPK activation assays

MAPK activation assays were performed on leaf discs from expanded leaves of 5-week-old plants or on 15-day-old seedlings 72 h post treatment with DMSO or estradiol. Samples were harvested 0, 5 mins, 15 mins, 30 mins and 45 mins post 100 nM flg22 treatment, and flash frozen in liquid nitrogen. Frozen samples were homogenized via 90 seconds of shaking (1100 rpm) in a GenoGrinder 2100, then 500 µL protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 10% glycerol, 0.5% NP40 IGEPAL® CA-630, protease inhibitor cocktail (Sigma) 1:100, phosphatase inhibitor (Sigma) 1:200, 1 mM $Na_2MoO_4 \times 2H_2O$, 1mM NaF, 1.5 mM activated Na_3VO_4, 5 mM DTT, 1mM PMSF) was added and the sample left to mix for 30 mins at 4 °C. Samples were centrifuged at max speed for 10 mins at 4 °C, supernatant transferred to a new tube and re-centrifuged at max speed for another 10 mins at 4 °C. The resulting supernatant was incubated at 95 °C for 15 mins with Laemmli buffer, then proteins were separated by SDS-PAGE and transferred to Immuno-blot® PVDF membrane. Proteins were detected using p44/42 MAPK (1:2000; Cell Signaling Technologies, #9102) and α-Rabbit-HRP (1:10000, Sigma, A0545).

ROS burst measurements

ROS assays were performed on leaf discs taken from 5-week-old plants or 15-day-old seedlings 72 h post DMSO or estradiol treatment. Leaf discs were first floated on H_2O overnight; the following day water was removed, then 20 µg/mL HRP and 6 µM L-012 was added, with or without 100 nm flg22. The same was performed for seedlings except liquid media was removed before adding the HRP and L-012. Chemiluminescence was recorded using a Varioskan Flash (Thermo Fisher) over 45 mins, with the total luminescence emitted used for analysis.

General ROS quantification

20 µM H_2DCFDA (Thermo Fisher) was syringe infiltrated into expanded leaves of 5-week-old plants 72 h post DMSO or estradiol treatment, and confocal microscopy was performed on the abaxial side of the leaf. Z-stacks were taken from four areas of a given leaf from four plants per genotype/treatment. Quantification of fluorescence was performed in FIJI using a sum projection.

RNAseq

Two mature leaves per 5-week-old plant were DMSO or estradiol treated, and harvested 12 h, 24 h, 48 h and 72 h post treatment. Each biological replicate contained four plants (i.e., 8 leaves), with three biological replicates for each genotype/treatment/time point. Leaves were flash frozen in liquid nitrogen, then homogenized via 90 seconds of shaking (1100 rpm) in a GenoGrinder 2100. Total RNA was extracted using the RNEasy Plant Mini Kit (QIAGEN, Germany) and eluted into 60 µl of water. Purified RNA was treated with the TURBO DNA-free kit (Thermo Fisher, USA). RNA quantification and library construction was conducted by Novogene Co., Ltd (Beijing). RNA quality and quantity was access via 1% agarose gel, NanoPhotometer ® spectrophotometer (IMPLEN, CA, USA), and the RNA Nano 6000 Assay Kit forthe Bioanalyzer 2100 System (Agilent Technologies, USA). Libraries were constructed using 0.4 µg of RNA and the NEBNext ® UltraTM RNA Library Prep Kity for Illumina (NEB, USA). mRNA was purified via poly-T magnetic beads. Fragmentation, cDNA synthesis, and NEBNext Adaptor hybridization were performed according to manufacturer's instructions and purified with the AMPure XP system (Beckman Coulter, USA). Index-coded samples were clustered on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, USA) and sequenced on an Illumina Novaseq platform to generate 150bp paired-end reads with

a sequencing depth of 10 million reads per sample. All sequencing data are available on GEO at GSE248301.

Bioinformatic analyses

Adaptors and low-quality (phred33<20) reads were trimmed from the data using TrimGalore v0.5.0 (Krueger et al. 2021) and Cutadapt v1.7 (Martin 2011). RNA quality was accessed using FastQC v0.11.8 (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data) and MultiQC v.1.7 (Ewels et al. 2016). The data was mapped against the *Arabidopsis* reference genome (TAIR10; NCBI Refseq GCA_000001735.1) using HISAT2 v2.1.0 (Kim et al. 2019). Mapped reads were sorted using Samtools v1.4.1 Sort (Danecek et al. 2021). Transcript assembly was completed using Stringtie v1.3.3 (Pertea et al. 2015) and gene counts (see Supplemental Data Set 9 and 10 for gene count matrix and metadata respectively) were extracted using the built-in python wrapper, prepDE.py available at http://ccb.jhu.edu/software/stringtie/dl/prepDE.py. Principle component analysis was conducted using DEseq2, ggplot2 (Wickham 2016), ggConvexHull, andTidyVerse (Wickham et al. 2019) in R (R Core Team 2022). Differentially expressed genes were determined using DEseq2 (Love et al. 2014) in R. Genes with less than 10 counts across all samples were excluded from the analysis. The data was subset by timepoints, and Wald Tests or Likelihood Ratio Tests (LRT) were performed based on comparisons of interest. Wald Tests were used to extract DEGs based on differences between treatment or genotype. LRT were used to extract genes differentially expressed based on difference between closed plasmodesmata (induced/estradiol treated LexA::PD-Plug and LexA::icals3m combined) and open plasmodesmata (uninduced/DMSO treated LexA::PD-Plug, uninduced/DMSO treated LexA::icals3m, and DMSO and estradiol treated Col-0). LRT was used in this way to determine genes uniquely up- or down-regulated in the inducible lines treated with estradiol, and not genes upregulated by estradiol secondary effects. Differentially expressed genes from the Wald Test were designated if the log2 fold change (lfc) was >1.5 or <-1.5 and adjusted p-value<0.05. Differentially expressed genes from the Likelihood Ratio Tests were considered significant if the adjusted p-value was less than 0.05. LRT significantly differentially expressed genes were clustered using Lasso2 (Lokhorst et al. 2021) and DEGreports v1.36.0 (Pantano 2023) and normalized read counts plotted

using ggplot2 and pheatmaps v1.0.12 (Kolde 2019). Genes involved in Salicylic Acid biosynthesis/catabolism and response were determined based on a Wald Test of open plasmodesmata versus closed plasmodesmata with a lfc cut-off of +/-0.5 and adjusted p-value of 0.05 (Supplemental Data Set 11). Gene ontology enrichment analysis was conducted based on differentially expressed genes. Enrichment was conducted in TopGo v2.52.0 (Alexa and Rahnenfuhrer 2023) based on TAIR10 GO terms, with a topgoFisher cut-off of $p<0.05$. GO terms were plotted in ggplot2. GO parent terms were determined using the Revigo's (Supek et al. 2011) redundancy feature (cut-off 0.7) and utilized to extract defense-specific enriched GO terms.

Transgene Mapping

Transgenes were mapped by generating a pseudo-genome consisting of the *Arabidopsis* TAIR genome as well as the transgene of interest (mCherry) using SeqKit v0.9.1 (Shen et al. 2016) and STAR v2.5a (Dobin et al. 2013). The reads were then mapped and sorted by coordinates to the pseudo-genome using STAR v2.5a. The data was processed the same as previously described using Samtools and Stringtie. Gene counts of mCherry (Supplemental Data Set 12) and CalS3 (Supplemental Data Set 13) were extracted using DESEQ2 and plotted in ggplot2.

Size phenotyping

9-day-old seedlings of Col-0, LexA::PD-Plug and LexA:: icals3m were finely sprayed with treatments DMSO or estradiol and 6 days later, the first two true leaves were adhered to a microscope slide and images taken on a Leica M205 FA stereo microscope. Leaf area was measured using FIJI.

Sugar and starch measurements

Leaf tissue was harvested in liquid nitrogen from plants 72 h post treatment of either DMSO or estradiol (3 leaves from 5-week-old plants), with sugars and starch separately extracted from approximately 100 mg total leaf tissue as described by (Smith and Zeeman 2006). Frozen tissue was homogenized for 2 mins at 1200 rpm in a GenoGrinder 2100. Samples were further homogenized in 1 mL of 0.7 M cold perchloric acid and then centrifuge at max speed 4 °C for 5 min. The supernatant was transferred to process for sugar, while the pellet was kept on ice for starch quantification. For sugar, 600 µL supernatant was transferred to a new 1.5 mL tube,

and neutralization buffer (2 M KOH, 400 mM MES) was added to achieve a pH of 6-7. The sample was then spun at max speed for 4 min and supernatant recovered. Soluble sugars were quantified by enzymatic assay in technical triplicates by monitoring NADH production absorbance at 340 nm in a 96-well format using an Omega FLUOstar microplate reader. Each sample was added to 50 µL of buffer (50 mM HEPES NaOH pH 7.4-7.6, 1 mM $MgCl_2$, 1mM ATP, 1 mM NAD, 1.4 U Hexokinase), topped with H_2O to a total volume of 198 µL. An initial A_{340} reading was taken, before adding glucose-6-phosphate dehydrogenase for a kinetic reading to determine glucose content, followed by adding phosphoglucose isomerase to determine fructose content, followed by invertase to determine sucrose content. To determine starch content, the sample pellet was first washed by adding 1 mL H_2O and vortexing, followed by centrifugation for 3 mins at 3,000 ×g. Supernatant was removed, then pellet washed again by adding 1 mL 80% ethanol and vortexing, followed by centrifugation for 3 mins at 3,000 ×g; this ethanol wash was repeated for a total of three washes. Excess ethanol was evaporated, then resuspended with 750 µL of H_2O. 2 × 200 µL of each sample was transferred to a new screw cap tube and incubated at 95 °C for 12 minutes. The samples were left to briefly cool to room temperature (~5 min), then one aliquot was digested by incubation with α-amylase (10 U) and amyloglucosidase (1.26 U) in 0.1045 M sodium acetate buffer (pH 4.8) for two hours at 37 °C, alongside the other aliquot serving as the non-digested control (i.e., containing only the 0.1045 M sodium acetate buffer and two hour 37 °C incubation). Starch content was quantified through enzymatic assay of the digested aliquot sample by monitoring NADH production as per the above glucose measurement. Technical triplicates were performed for each digested sample, alongside a single technical replicate for the non-digested sample. Starch content was calculated from the glucose assay values in mg per gram of fresh weight of sample.

Salicylic acid measurements

Leaves from 5-week-old plants were treated with DMSO or estradiol and harvested 72 h post treatment and flash frozen in liquid nitrogen; per biological replicate, a minimum of six leaves from a minimum of three plants were pooled together to obtain between 150-220mg of fresh tissue weight. Frozen tissue was homogenized for 2 × 2 mins at 1200 rpm in a GenoGrinder 2100. 300 µL of buffer (10% methanol, 1% acetic acid in H_2O) containing an internal standard of 1 µM salicylic acid-d_4 (SA-$_{d4}$, Merck) was

added, mixed well with a vortex then left to mix at 4 °C for 20 mins. Samples were centrifuged for 25 mins at 4°C at 15,000 RCF, and the supernatant recovered. The pellet was re-extracted with another 300 µL buffer without the internal standard, mixed and centrifuged as before, with the resulting supernatant combined with the first recovered supernatant. The total supernatant was centrifuged at 4 °C for 10 mins at max speed, and each sample was then run for analysis. Quantification was performed on an Acquity UPLC attached to a Xevo TQS tandem mass spectrometer (Waters). Separation was on a 50×2.1mm 2.6µM Kinetex EVO C18 column (Phenomenex) using the following gradient of acetonitrile (solvent B) versus 0.1% formic acid in water (solvent A), run at 600 µL.min-1 and 35°C: 0 min, 10% B; 2 min, 80% B; 2.15 min, 80% B; 2.2 min, 10% B; 3.4 min, 10% B. The injection volume was 5 µL. Salicylate was quantified by negative mode electrospray, monitoring the transition *m/z* 137→93 (and for the internal standard the corresponding transition 141→97) at 16V collision energy. Spray chamber conditions were 1.5kV spray voltage, 500°C desolvation temperature, 900 $L.hr^{-1}$ desolvation gas, 150 $L.hr^{-1}$ cone gas, and 7 bar nebulizer pressure. Results were processed using TargetLynx software V4.1 SCN876 (2012).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were performed using an Imaging-PAM chlorophyll fluorometer and ImagingWin software application (Walz). The effective PSII quantum yield as indicated by Y(II) was taken following a 30 min dark adaptation of leaves from 5-week-old plants 72 h post DMSO or estradiol treatment. Four leaves per treatment/genotype were measured in a given run, with 8 runs in total over two independent experiments.

Statistical methods

Statistical analyses were performed using RStudio 2021.09.01 Build 351/R version 4.0.3. Data presented as boxplots have the middle line indicating the median, the box representing the upper and lower quartiles, and the whiskers indicating the minimum and maximum values with 1.5× interquartile range. For bootstrap analyses, data were analyzed using *medianBootstrap* (Johnston and Faulkner 2021) or *medianBootstrap2* (Tee et al. 2023a). Pathoassays, basal ROS quantification, and chlorophyll fluorescence measurements were analyzed using a linear-mixed effects model using the R package, *lmerTest*. For pathoassays the random effect was ‘independent

experiment repeat'; for the basal ROS quantification the random effect was 'biological replicate'; for chlorophyll fluorescence measurements the random effect was 'experimental run'. Size phenotyping, starch, sucrose, glucose and fructose measurements were analyzed using a linear model. All ANOVAs specified are ANOVA Satterthwaite's Method, and significant differences between factors were determined by post hoc Tukey HSD using the R package, *emmeans*. Salicylic acid quantification was analyzed using a Mann-Whitney test.

Accession Numbers

Sequence data from this article can be obtained from in the GenBank/European Molecular Biology Laboratory databases under the following accession number: *CalS3* (AT5G13000).

Supplementary Data sets

Supplemental Data Set 1: Data for number of cells showing GFP within a genotype/treatment

Supplemental Data Set 2: Background response to estradiol: differentially regulated genes in response to estradiol treatment shared across all genotypes

Supplemental Data Set 3: Genes differentially expressed in response to 12h plasmodesmal closure identified by a likelihood ratio test analysis

Supplemental Data Set 4: Genes differentially expressed in response to 24h plasmodesmal closure identified by a likelihood ratio test analysis

Supplemental Data Set 5: Genes differentially expressed in response to 48 h plasmodesmal closure identified by a likelihood ratio test analysis

Supplemental Data Set 6: Genes differentially expressed in response to 72h plasmodesmal closure identified by a likelihood ratio test analysis

Supplemental Data Set 7: Genes identified from the literature as associated with salicylic acid biosynthesis, catabolism and response

Supplemental Data Set 8: Genes identified from the literature as associated with the synthesis and transport of soluble sugars and starch

Supplemental Data Set 9: Gene count matrix from the RNAseq analysis

Supplemental Data Set 10: Metadata related to the gene count matrix

Supplemental Data Set 11: Log2 fold change of genes associated with SA that are differentially expressed at 12h, 24h, 48h and 72h

Supplemental Data Set 12: Gene counts of mCherry from pseudo-genome GEO GSE248301

Supplemental Data Set 13: Gene counts of CalS3 from pseudo-genome GEO GSE248301

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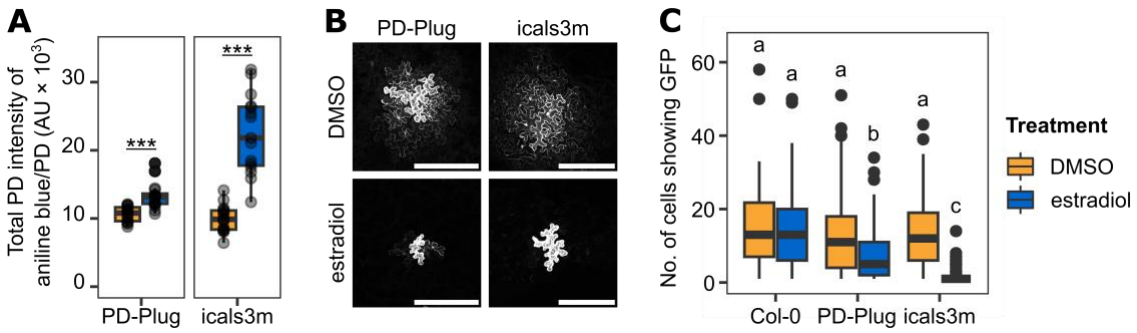

Figure 1. Characterization of plasmodesmal status in induced LexA::PD-Plug and LexA::icals3m tissue. A) Quantification of aniline blue-stained plasmodesmata-associated callose in 5-week-old plants of LexA::PD-Plug (PD-Plug) or LexA::icals3m (icals3m) 24 h post DMSO or estradiol treatment. Datapoints represent the average of the total PD intensity/plasmodesmata (PD) per image, with n ≥ 14 per genotype/treatment. Bootstrap analysis indicates significant differences between DMSO and estradiol treatment in PD-Plug and icals3m, as indicated by *** $p<0.001$. **B)** Example micrographs of bombardment sites showing movement of GFP from transformed cells indicate cell-to-cell connectivity of uninduced (DMSO) versus restricted cell connectivity in induced (estradiol) leaves of PD-Plug and icals3m. **C)** Quantification of microprojectile bombardment data showing GFP movement into neighboring cells. Leaves of 5-week-old plants were bombarded after 24 h treatment of DMSO or estradiol; n ≥ 62 bombardment sites. Bootstrap analysis with significant differences indicated by a, b and c, with $p<0.01$. Scale bar = 200 µM.

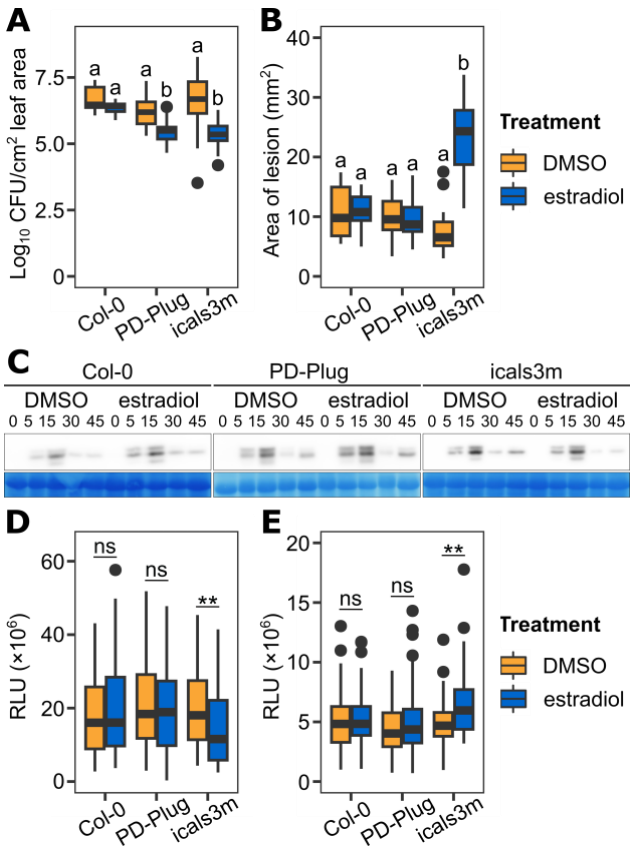

Figure 2. Plasmodesmal closure differentially impacts pathogen resistance and immune responses. A) Bacterial growth ($\log_{10}$ CFU/cm^2 leaf area) of *Pst* DC3000 3 dpi on uninduced and induced tissue of 4–5-week-old plants of Col-0, LexA::PD-Plug (PD-Plug) and LexA::icals3m (icals3m). Tissue was treated with DMSO (uninduced) or estradiol (induced) 72 h prior to infection. 4 independent experiments were conducted, with 5 plants per treatment/genotype; N = 20. Independent factors genotype and treatment were significant, with a significant interaction between the two (ANOVA; F = 16.6, df = 2, P < 0.0001; F = 67.0, df = 1, P < 0.0001; F = 8.2, df = 2, P < 0.001). Significant differences denoted by a and b, p < 0.0001. **B)** The average area of *Botrytis cinerea* disease lesions 2 dpi in leaves of 5-week-old plants of induced and uninduced Col-0, LexA::PD-Plug and LexA::icals3m. Tissue was treated with DMSO (uninduced) or estradiol (induced) 72 h prior to infection, with N = 16-17 for all genotypes/treatment combined from three independent repeats. Independent factors genotype and treatment were significant with a significant interaction between the two (ANOVA; F = 24.4, df = 2, P < 0.0001; F = 52.7, df = 1, P < 0.0001; F = 57.0, df = 2, P < 0.0001). Significant differences denoted by a and b, p < 0.0001. **C)** Western blot detection of MAPK-activation by flg22 treatment in 4–5-week-old plants of Col-0, LexA::PD-Plug and LexA::icals3m 72 h post DMSO or estradiol treatment. Minute timings of tissue harvest are denoted, and Coomassie blue staining shown below as loading controls. **D)** Average relative light units (RLU) generated from ROS production in response to flg22 in leaf discs of 5-week-old plants. Leaves were treated with estradiol or DMSO 72 h prior to flg22 treatment and data is combined from two independent experiment with N = 95 per genotype/treatment. Significant differences between treatment determined by bootstrap analysis, with significant differences denoted by **, p < 0.01. **E)** Average RLU generated from ROS production in response to flg22 in seedlings. Plants were treated with estradiol or DMSO 72 h prior to flg22

981 treatment, with N ≥ 80 per genotype/treatment. Significant differences between
982 treatment determined by bootstrap analysis, with significant differences denoted by **,
983 $p < 0.01$.

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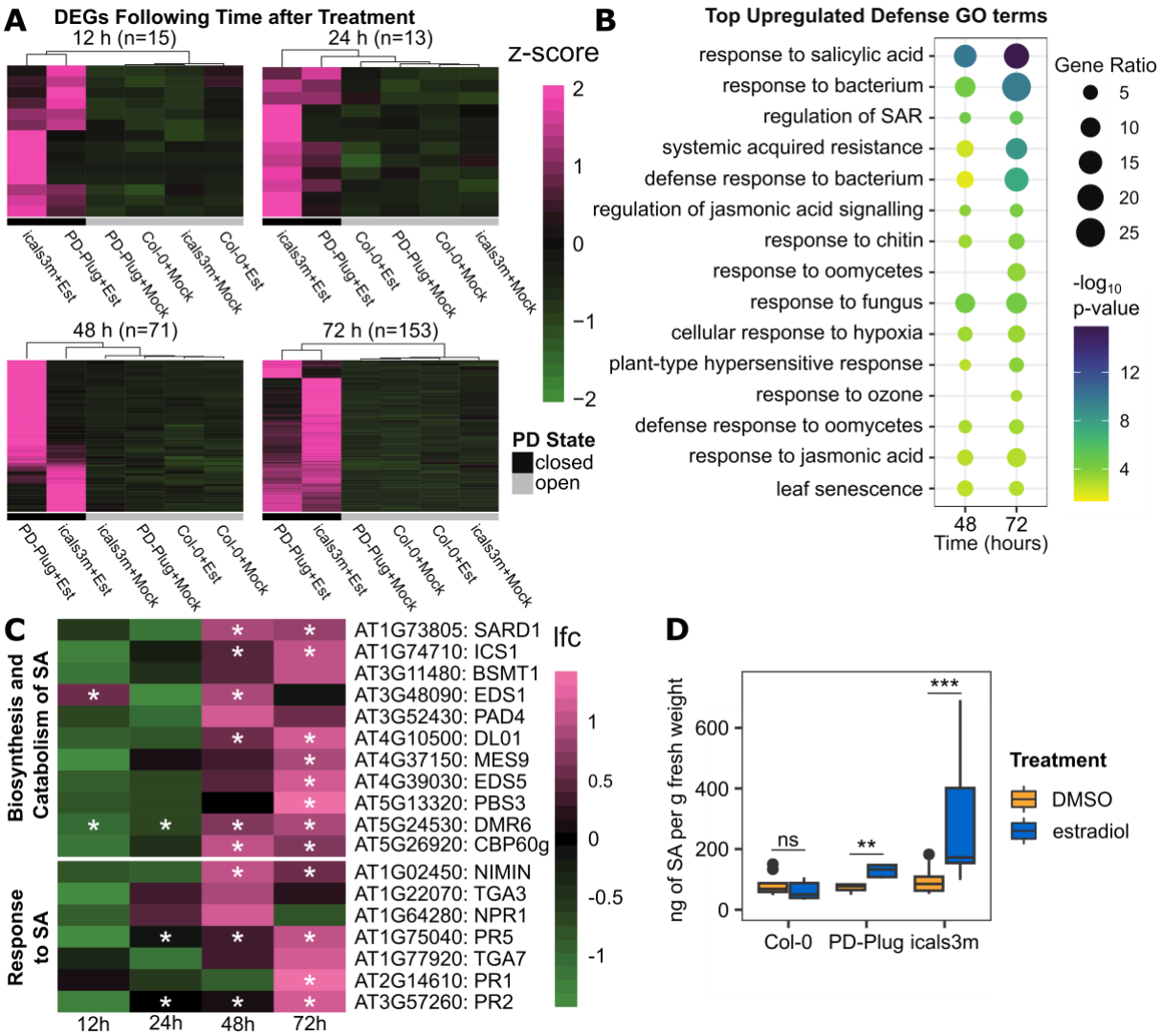

Figure 3. Plasmodesmal closure triggers stress transcriptional responses and salicylic acid related changes. A) Likelihood ratio test analysis determined number (indicated by n) of differentially expressed genes (DEGs) significantly differed between plasmodesmal status (i.e., PD State closed or open), and matches the cluster pattern of upregulated in closed state in comparison to open state at 12, 24, 48 and 72 h post treatment. Hierarchical clustering grouped together plasmodesmal state of open (i.e., estradiol treated LexA::PD-Plug and LexA::icals3m) and closed (DMSO treated LexA::PD-Plug, LexA::icals3m and Col-0, and estradiol treated Col-0). Expression normalized by row (z-score). **B)** Top 15 defense GO terms enriched over time when plasmodesmata are closed. **C)** Expression of genes related to the salicylic acid biosynthesis/catabolism and response over time when plasmodesmata are closed (i.e., in estradiol treated LexA::PD-Plug and LexA::icals3m). **D)** Quantification of salicylic acid in 5-week-old plants after 72 h DMSO or estradiol treatment, n ≥ 6 per genotype/treatment. Significant differences between treatment within a genotype analyzed using Mann-Whitney test denoted by **, $p < 0.01$, or ***, $p < 0.001$.

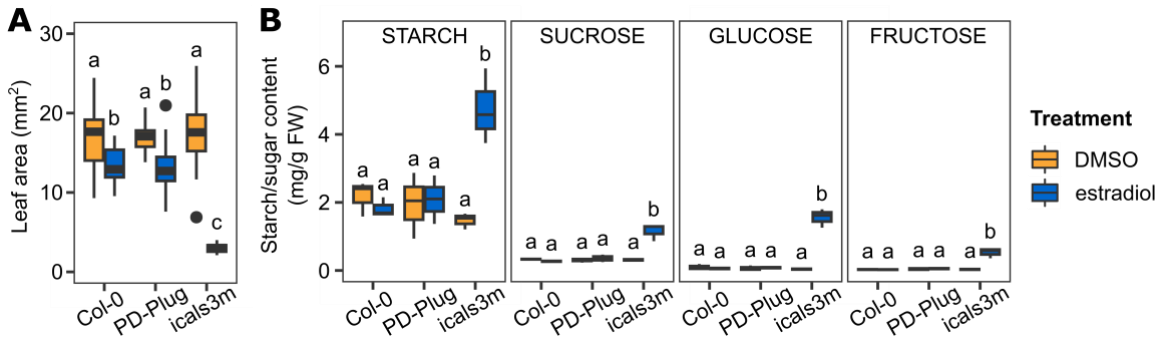

Figure 4. Plasmodesmal closure induces sugar accumulation and impairs leaf growth. A) Leaf area of first two true leaves 6 days post treatment (DMSO or estradiol). Independent factors genotype and treatment were significant, with a significant interaction between the two (ANOVA; F = 82.4, df = 2, P < 0.0001; F = 445.0, df = 1, P < 0.0001; F = 104.2, df = 2, P < 0.0001). Significant differences denoted by a, b and c, p < 0.0001. n = 40. **B)** Starch, sucrose, glucose and fructose content in leaves of 5-week-old plants 72 h post DMSO or estradiol treatment. Independent factors genotype and treatment were significant, with a significant interaction between the two for each metabolite (ANOVA; *starch* F = 4.7, df = 2, P < 0.05, F = 9.2, df = 1, P < 0.05; F = 11.4, df = 2, P < 0.01; *sucrose* F = 26.2, df = 2, P < 0.0001; F = 25.8, df = 1, P < 0.001; F = 26.7, df = 2, P < 0.0001; *glucose* F = 70.0, df = 2, P < 0.0001; F = 74.4, df = 1, P < 0.0001; F = 79.3, df = 2, P < 0.0001; *fructose* F = 28.0, df = 2, P < 0.0001; F = 30.4, df = 1, P < 0.0001; F = 28.8, df = 2, P < 0.0001). Significant differences denoted by a and b, p < 0.01.

Table 1. Photosynthetic yield as indicated by Y(II) following DMSO or estradiol treatment of leaves of Col-0, LexA::PD-Plug (PD-Plug) and LexA::icals3m (icals3m). Pooled average of Y(II) ± SD, with a biological replicate data averaged from 4 leaves per treatment/genotype on a given experimental run where N = 8, combined from two independent experiments. Independent factors genotype and treatment were not significant, with no significant interaction between the two (ANOVA; F = 1.3, df = 2, P = 0.29; F = 1.2, df = 1, P = 0.29, F = 0.1, df = 2, P = 0.92).

	Col-0	PD-Plug	icals3m
DMSO	0.79±0.02	0.78±0.02	0.78±0.02
estradiol	0.79±0.01	0.78±0.01	0.79±0.01