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Blue light induces a distinct starch degradation pathway in guard cells for stomatal opening

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SUMMARY

Stomatal pores form a crucial interface between the leaf mesophyll and the atmosphere, controlling water and carbon balance in plants [1]. Major advances have been made in understanding the regulatory networks and ion fluxes in the guard cells surrounding the stomatal pore [2]. However, our knowledge on the role of carbon metabolism in these cells is still fragmentary [3–5]. Particularly, the contribution of starch in stomatal opening remains elusive [6]. Here, we used *Arabidopsis thaliana* as a model plant to provide the first quantitative analysis of starch turnover in guard cells of intact leaves during the diurnal cycle. Starch is present in guard cells at the end of night, unlike in the rest of the leaf, but is rapidly degraded within 30 min of light. This process is critical for the rapidity of stomatal opening and biomass production. We exploited *Arabidopsis* molecular genetics to define the mechanism and regulation of guard cell starch metabolism, showing it to be mediated by a previously uncharacterized pathway. This involves the synergistic action of β-amylase 1 (BAM1) and α-amylase 3 (AMY3) – enzymes that are normally not required for night-time starch degradation in other leaf tissues. This pathway is under the control of the phototropin-dependent blue light signalling cascade and correlated with the activity of the plasma membrane H^+-ATPase. Our results show that guard cell starch degradation has an important role in plant growth, by driving stomatal responses to light.

RESULTS

Starch dynamics in guard cells of intact *Arabidopsis* leaves

Starch is a complex glucose polymer found in plastids of higher plants [7]. In guard cell chloroplasts, starch has long been implicated in light-induced stomatal opening. Outlaw and Manchester showed 35 years ago that guard cell starch concentration was higher in leaflets of *Vicia faba* with closed stomata than with open stomata [8]. Schnabl showed a correlation between changes in volume of *Vicia faba* guard cell protoplasts and proposed the interconversion of malate and starch [9]. Talbott and Zeiger observed an increase in sucrose levels in *Vicia faba* epidermal peels throughout the light-stimulated stomatal opening concomitantly with the accumulation of the starch hydrolytic products maltose and maltotriose [10]. These observations led to the hypothesis that guard cell starch is mobilized in the light to generate malate and/or sucrose to sustain stomatal opening. After these early publications, the literature on the subject has been fragmentary and mostly correlative in nature, due to the lack of suitable methods and genetic material. Changes in guard cell starch concentrations were observed in other species (*Ocimum basilicum* and *Commelina communis*), but no details about the temporal dynamics nor the metabolic pathway were reported [4, 11]. In *Arabidopsis thaliana* – the most well studied model plant – there is no clear evidence of starch breakdown in guard cells in the light. Lasceve *et al.* showed electron micrographs of starch granules in *Arabidopsis* guard cells at the end of the night [12], suggesting that starch metabolism follows an opposite rhythm compared to mesophyll cells, where starch is degraded at night [13]. In contrast, Stadler and coworkers reported that starch was nearly absent in *Arabidopsis* guard cells at the end of the night [14]. Recently, Daloso *et al.* suggested that in tobacco guard cells changes in sugars – not starch – drive light-induced stomatal opening [15]. Due to these contradictory reports, guard cell starch metabolism is

currently thought to differ between species [6] and its genetic and molecular basis remains mostly unknown.

Until now, starch content in guard cells has been mostly estimated on a relative value-scale by comparing the intensity of iodine-stained guard cell chloroplasts [11, 16–18]. A few studies have determined guard cell starch content quantitatively using the "oil well technique" on freeze-dried leaflets [8, 19], or spectrophotometrically using guard cell-enriched epidermal fragments [15]. In such cases, however, no details of temporal dynamics over the diurnal cycle were provided. Here, we developed a new method to quantify starch in guard cells of intact leaves. Epidermal peels from mature leaves were immediately fixed, treated with periodic acid, and the glucans covalently linked to the fluorophore propidium iodide. Confocal microscopy then enabled high-resolution analytical imaging to quantify the starch in guard cells over the diurnal cycle. This analysis was performed in *Arabidopsis* because this plant is widely used as a model system for guard cell physiology, because of the contradictory literature regarding its guard cell starch metabolism, and to exploit the wealth of molecular genetic resources. As for other species [4, 8, 11], starch was present in *Arabidopsis* wild-type (WT) guard cells at the end of the night (Figures 1A-1C). In response to light, starch was rapidly degraded within an hour (Figures 1A-1C). As expected, guard cells of the *Arabidopsis* starch deficient mutant *pgm* (*PHOSPHOGLUCOMUTASE 1*) were devoid of starch (Figure 1A)[12]. Interestingly, in WT plants, starch started to decline during the latter part of the night and by dawn about half was gone. However, night-time degradation occurred slowly compared to the light-induced degradation (Figure 1B). Starch synthesis started 1 h into the light, was maintained for the rest of the photoperiod and continued into the night (Figure 1B). These results show that *Arabidopsis*

guard cell starch metabolism differs markedly from the rest of the leaf, where starch is synthesized during the day and degraded at night in a near-linear manner [20].

BAM1 and BAM3 have a cell type-specific function

Next, we investigated the enzymes responsible for starch degradation in guard cells. β-amylase (BAM), which releases maltose from glucans, is the main starch-degrading enzyme [7, 13]. Mutant studies suggest that BAM3 is the major isoform in leaf mesophyll starch metabolism, while BAM1 has limited or no involvement [21]. Recently, it was shown that *bam1* mutants have more starch in illuminated guard cells and reduced stomatal opening compared to WT [18], which resulted in drought tolerance [17]. Consistently, we found that *BAM1* was preferentially and highly expressed in guard cell-enriched epidermal peels, similarly to established markers for guard cell-specific expression; the inward-rectifying *K^+ CHANNEL IN ARABIDOPSIS THALIANA 1* (*KAT1*), *CYTOCHROME P450 86A2* (*CYP86A2)* and *MYB TRANSCRIPTION FACTOR 60* (*MYB60)* (Figures 1D and 1E)[22, 23]. Compared with WT, *bam1* mutants displayed a guard cell-specific *s*tarch *ex*cess (*sex*) phenotype throughout the diurnal cycle (Figures 1A-1C, and S1). To provide direct genetic evidence that the mutation in *BAM1* was responsible for the guard cell *sex* phenotype, we repressed *BAM1* in guard cells of WT using microRNA-induced gene silencing (MIGS)[24], driven by the *CYP86A2* promoter [23]. *BAM1* silencing lines showed reduced guard cell *BAM1* transcript levels and increased starch content, mimicking the *bam1* phenotype (Figures 1A, 1C and 1E). These results show that, under standard growth conditions, BAM1 functions specifically in guard cell starch metabolism. In contrast, *BAM3* transcripts were considerably less abundant in guard cells (Figure 1D) and guard cell starch levels in *bam3* were similar to WT (Figures 1A-1C). This suggests that BAM1 and

BAM3 acquired different cell type-specific functions. Interestingly, *bam1bam3* double mutants had similar guard cell starch content to the *bam1* single mutants (Figures 1A and 1C). This contrasts to the situation in mesophyll cells, where *bam1bam3* has a more severe *sex* phenotype than *bam3* single mutants (Figure S1)[21]. These data reveal that sub-functionalization amongst the chloroplastic BAMs enables the plant to adjust starch turnover to the needs of the individual cell.

A distinct pathway of starch degradation operates in guard cells during light-induced stomatal opening

Given the complex nature of starch polymers, β-amylases need to work with other glucan hydrolases to accomplish complete degradation [13, 25, 26]. We hypothesized that other enzymes that have no clear role in mesophyll starch metabolism may have a role in guard cells. As for BAM1, loss of *α-AMYLASE 3* (*AMY3*) and the debranching enzyme *LIMIT DEXTRINASE* (*LDA*) do not affect starch metabolism in the leaves (Figure S1)[27, 28]. Interestingly, *AMY3* was strongly up-regulated in guard cells (Figure 2A) and the *amy3* mutant showed a slight but significant and reproducible guard cell *sex* phenotype (Figures 2B and 2C). Loss of both AMY3 and BAM1 proteins resulted in a much higher starch content than in the *bam1* mutant (Figures 2B and 2C), without affecting starch metabolism in the leaves (Figure S1). This striking *amy3bam1* phenotype indicates a role for AMY3 in guard cell starch metabolism. Unlike *AMY3* and *BAM1*, *LDA* was not up regulated in guard cells (Figure 2A). Mutation of *LDA* had a marginal, albeit significant and reproducible impact on guard cell starch metabolism (Figures 2B and 2C). However, transcript abundance of *ISOAMYLASE 3* (*ISA3*) - the major debranching enzyme in night-time leaf starch degradation – was 7-fold higher in guard cell-

enriched epidermal peels relative to intact leaves (Figure 2A). Guard cells of *isa3* contained elevated starch compared with WT, even though appreciable starch degradation still occurred upon illumination (Figures 2B and 2C). The *isa3* phenotype was exacerbated by the additional mutation of LDA (*isa3lda*, Figures 2B and 2C), reproducing in guard cells the situation previously described in leaves (Figure S1)[28]. Collectively, these results indicate that BAM1, AMY3, LDA and ISA3 work together towards efficient guard cell starch mobilization during stomatal opening.

Mutations in the stomatal starch degradation pathway impact plant growth

To investigate the importance of starch degradation in guard cells, we examined the consequences of the absence of BAM1 and AMY3 on stomatal function and whole-plant physiology. Intact leaves of *bam1* mutants showed reduced stomatal aperture and slower increases in stomatal conductance (g_s) in response to light compared with WT (Figures S2A and S2B). This correlates with the lack of light-induced starch degradation in *bam1* guard cells (Figures 1A-1C). However, CO_2 assimilation (A) was generally not affected in *bam1* plants, with only subtle differences during the first 1 h of light (Figure S2C). In contrast to *bam1*, *bam3* mutants had normal rate of stomatal opening in response to light. Transpiration and carbon assimilation rates were also normal (Figures S2D-S2F) indicating that BAM3 is not required for proper stomatal function.

The additional loss of AMY3 exaggerated the suppression of plant stomatal responses. Firstly, *amy3bam1* plants failed to open stomata efficiently in response to light (Figure 3A). Consequently, *amy3bam1* had lower g_s (Figure 3B), decreased leaf intercellular CO_2 concentration (C_i; Figure 3C) and slightly lower CO_2 assimilation rates (Figure S2G)

immediately after illumination, compared with WT. This suppression of stomatal function in the *amy3bam1* double mutant had consequences for plant growth, especially under higher light intensities. 4-week-old *amy3bam1* plants grown under 120 µmol m^{-2} s^{-1} of light were almost 30% smaller than WT, whereas there were no noticeable differences when plants were kept under low light intensities (Figure 3D and Figure S2H). These results suggest that diffusive (stomatal) limitations in *amy3bam1* plants may limit CO_2 availability for mesophyll photosynthesis upon illumination, at least under higher light intensities. Thus, we conclude that guard cell starch degradation is of major importance in plant adaptation to light through the control of stomatal aperture.

Guard cell starch degradation is specifically activated by blue light through the PHOT1/PHOT2-dependent signaling pathway

Starch degradation in guard cell chloroplasts is believed to occur mainly under blue light [5, 10], as the *Arabidopsis pgm* mutant shows a reduced rate of stomatal opening, which seems largely attributable to the inhibition of blue light-induced mechanisms [12]. Consequently, we investigated whether blue light is the actual signal for guard cell starch degradation. We found that starch was efficiently degraded and stomata opened in *Arabidopsis* WT plants illuminated with 75 µmol m^{-2} s^{-1} of blue light, a stimulus insufficient to activate photosynthesis at significant levels (Figures 4A, S3A and S3B). In contrast, illumination with 300 µmol m^{-2} s^{-1} of red light promoted efficient starch synthesis in guard cells and induced stomatal opening independently of starch (Figures 4A and S3A). Thus, distinct light qualities control synthesis and degradation of starch in guard cells, and stomatal opening triggered by red light seems to be largely independent of the breakdown of starch present in guard cell chloroplasts.

In guard cells, blue light perception by *PHOTOTROPIN 1* and *2* (*PHOT1/PHOT2*) [29] initiates a signal transduction cascade, involving the *BLUE LIGHT SIGNALING 1* (*BLUS1*) protein kinase [30] and the *PROTEIN PHOSPHATASE 1* (*PP1*) [31, 32]. The signal ultimately activates the plasma membrane H^+-ATPase, leading to increased inside-negative electric potential, influx of potassium and water, and opening of the stomatal pore [2, 33–35]. To determine the molecular basis of blue light-induced guard cell starch degradation, we investigated starch turnover in the *Arabidopsis* blue light-signaling mutants *phot1phot2* and *blus1*. In both mutants, guard cells of intact leaves still contained high levels of starch after 1 h of light, whereas WT and the *blus1::GFP-BLUS1* complementing line efficiently degraded it (Figures 4B, 4C, S3C and S3D). In fact, WT consumed almost all the starch within 30 min of light (Figure 4B), showing that the response of guard cell starch degradation to light is extremely rapid. In contrast to guard cells, synthesis and degradation of starch in *phot1phot2* and *blus1* mesophyll cells occurred normally (Figures S3E and S3F), even though the overall starch levels in *phot1phot2* leaves were reduced compared to WT (Figure S3E). This is most likely a consequence of the impaired CO_2 assimilation rate in this mutant [36]. Tautomycin is a potent inhibitor of PP1, which mediates blue light signaling between phototropin and the plasma membrane H^+-ATPase [32]. Consistent with a role for blue light signaling in guard cell starch degradation, we found that tautomycin completely blocked starch degradation and stomatal opening (Figure 4D and S3G). Altogether, these results indicate that the effect of blue light on starch degradation is phototropin-dependent and specific to guard cells.

Proton extrusion by H^+-ATPase is required for starch degradation during stomatal opening

Given that the plasma membrane H^+-ATPase is the ultimate target of the blue light signaling cascade [33], we investigated if proton pump activity was required for guard cell starch breakdown. Mutation of plasma membrane H^+-ATPase1 (*aha1*) resulted in a severe guard cell-*sex* phenotype, without affecting leaf starch metabolism (Figures 4C and S3F). In contrast, mutants of H^+-ATPase2 (*aha2*) displayed a WT phenotype (Figure 4C), suggesting a major role for AHA1 in guard cells, although both isoforms are expressed [37].

The fungal toxin fusicoccin (Fc) is a well-known chemical activator of the proton pump [38]. As expected, stomata in WT isolated epidermal peels floating in a buffer containing 50 mM KCl opened to a greater extent in the presence of 10 µM Fc (Figure 4E)[29]. Starch in guard cell chloroplasts in isolated epidermal peels floating in control buffer showed a similar pattern of light-induced starch degradation and re-synthesis to that of guard cells of intact leaves (Figure 4F vs 1C). However, upon treatment with Fc, almost all guard cell starch disappeared after 1 h (Figure 4F), suggesting that enhancement of the H^+-ATPase activity correlates with enhanced starch degradation. A similar pattern of stomatal opening and guard cell starch accumulation was found in *phot1phot2* following Fc treatment (Figures 4E and 4F). On the basis of these results, we conclude that proton extrusion across the plasma membrane during stomatal opening is required for guard cell starch breakdown and that the activity of the H^+-ATPase is positively correlated with starch degradation.

Stomata in *bam1* isolated epidermal peels floating in the buffer containing 50 mM KCl opened similarly to that of WT, most likely due to the presence of high concentration of Cl^- in the medium (Figure 4E). The presence of Cl^-, however, had only little effect on *amy3bam1* stomatal

opening (Figure 4E). Exogenous application of Fc only partially rescued stomatal opening and guard cell starch degradation in *bam1*, and had little or no effect on *amy3bam1* (Figures 4E and 4F). These results indicate that H^+-ATPase functions upstream of starch degradation and that this process is ultimately required for blue light-induced stomatal opening.

DISCUSSION

It has been 100 years since the first observation that starch metabolism in guard cell chloroplasts follows a different rhythm compared to other photosynthetically active leaf tissues [39]. However, despite the increasing interest in guard cell function and regulation, the role of starch metabolism in guard cells and its influence on stomatal function remains only poorly understood, especially in *Arabidopsis*. Our new protocol to quantify starch in single cells showed that starch is present in *Arabidopsis* guard cells at the end of the night, unlike the rest of the leaf. Upon illumination, the starch is very rapidly degraded and most is gone within 30 min (Figures 1B and 4B). Thus, we conclude that starch metabolism in *Arabidopsis* guard cells is similar to that of other species [4, 8, 11], overcoming the contradictions in the literature [12, 14].

By conducting *Arabidopsis* mutant analyses, we discovered that light-induced starch degradation in guard cells is surprisingly mediated by a previously uncharacterized pathway, which involves the activity of BAM1 and AMY3, which are normally not required for night-time starch degradation in the rest of the leaf (Figures 1, 2 and S1). We propose that a key mechanism underpinning such plasticity in starch mobilization is enzyme isoform specialization. In support of this, we showed that two β-amylase isoforms have acquired tissue-specific function. BAM3 has low expression in guard cells (Figure 1D) but represents the major isoform in the night-time leaf starch degradation (Figure S1)[21]. BAM1 is highly expressed in guard cells and is required

for light-induced starch degradation in these cells (Figure 1 and S1)[18]. Interestingly, BAM1 is redox regulated, whereas BAM3 is not, and the cysteines involved in the disulfide formation in BAM1 are not conserved in BAM3 [40, 41]. It is therefore plausible to imagine that redox control of BAM1 may activate starch degradation in the light when the cellular environment becomes more reducing due to the activity of the electron transport chain [42]. We recently showed that, similarly to BAM1, AMY3 is also a redox regulated enzyme [26], strengthening the concept that BAM1 and AMY3 are co-regulated and work synergistically. Blocking starch degradation in guard cells by simultaneous loss of BAM1 and AMY3 proteins has severe consequences for stomatal function and a perceptible impact on plant growth, especially under high light intensities (Figure 3). This knowledge reveals the importance of starch in guard cells in driving plant growth through the control of stomatal movements.

Lastly, we provide compelling evidence that this newly discovered starch degradation pathway is under the direct control of blue light through the PHOT1/PHOT2-dependent signaling cascade. Mutation in the blue light-signaling components PHOT1/PHOT2 or BLUS1 impacted starch degradation in guard cells (Figures 4B and 4C), but not in mesophyll cells (Figures S3E and S3F), demonstrating that the influence of blue light on starch metabolism is specific to guard cells. Furthermore, we provide evidence of a direct link between proton pumping and starch degradation in guard cells. The inability of the *aha1* mutants to mobilize starch in guard cells efficiently upon illumination (Figure 4C), along with the low sensitivity of *amy3bam1* guard cells to Fc-treatment (Figures 4E and 4F), show that proton pumping is required for starch degradation during stomatal opening. Interestingly, a previous study showed a similar correlation between proton pumping and the activity of the guard cell isoform of *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PEPC*), which is required for the synthesis of

malate [43]. This observation points towards a coordinated activation of the blue light-dependent mechanisms required for stomatal opening, whereby maltose originated from starch degradation is converted to malate in the cytosol through glycolysis and the action of PEPC, with an estimated net production of 4 molecules of malate and one molecule of ATP per molecule of maltose (S1 Data). Considering the malate concentration of open guard cells to be around 100-200 mM [44], we propose that approximately half of the starch broken down during early stomatal opening is sufficient for rapid malate synthesis (S1 Data). The fate of the remainder carbon skeletons is not clear, but presumably used for sucrose or other hexose sugars production as additional osmolytes or for cellular respiration to produce the ATP required for H^+ pumping.

The signal ultimately linking plasma membrane H^+-ATPase activity to starch degradation in the chloroplast is unknown. The alkalinization of the cytosol resulting from proton pumping upon illumination stimulates accumulation of H^+ through malate synthesis [45], a process requiring carbon skeletons deriving from starch degradation [9, 46]. Depletion of protons in the cytosol may therefore represent a signal for guard cell starch degradation in response to light, although it is difficult to image how changes in cytosolic pH are transferred to the chloroplast. Alternatively, changes in metabolic fluxes upon activation of stomatal opening may trigger starch degradation indirectly, through metabolic signals such as the increase in ADP:ATP ratio or the depletion of the glycolysis intermediate PEP. Lastly, both BAM1 and AMY3 are preferentially active at a slightly alkaline pH [26, 41, 47]. Thus, the reducing environment and the alkaline pH of the stroma generated by the photosynthetic electron transport chain in the light [42, 48], would favor BAM1 and AMY3 activity, promoting starch degradation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, S1 Data file, three figures and one table.

AUTHOR CONTRIBUTIONS

D.S. conceived the project. D.S. and D.H. designed the experiments. D.H., S.F., D.P., JSA. M., M.T., and A.N. performed the experiments and analyzed the data. D.S., D.H., and T.L. interpreted the data. N.L. contributed new reagents/analytic tools. D.S. and D.H. wrote the paper.

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FIGURE LEGENDS

Figure 1. Starch turnover in *Arabidopsis* guard cells and isoform specialization within the β-amylase family. (A) Visualization of starch granules within *Arabidopsis* guard cells of intact leaves at the end of the night (EoN) and after 1 h and 3 h of illumination. Scale bar = 10 µm. (B) Starch accumulation in guard cells of intact leaves over the diurnal cycle (n = 110 ± sem). (C) Starch accumulation in guard cells of intact leaves at the EoN and after 1 h and 3 h of illumination (n = 100 ± sem). Unpaired Student's t test determined statistical significance between EoN and 1 h light (* $p < 0.05$; n.s. not significant). (D) Gene transcript abundance in guard cell-enriched epidermal peel fragments compared to leaves. Values are normalized against gene expression in the leaves (set as 0; n = 3 ± sem). (E) Relative expression levels of *BAM1* in leaves and guard cell-enriched epidermal peel fragments of WT and two independent *BAM1* MIGS silencing lines (n = 3 ± sem). *BAM1* transcripts in the guard cells of the two silencing lines are reduced by 60% and 80%, respectively, but are unaffected in the leaves. See also Figure S1.

Figure 2. BAM1, AMY3, ISA3 and LDA form a distinct pathway of starch degradation in *Arabidopsis* guard cells. (A) Transcript abundance of *AMY3*, *ISA3* and *LDA* in guard cell-enriched epidermal peel fragments compared to leaves. Values are normalized against gene expression in the leaves (set as 1; n = 3 ± sem). (B) Quantification of starch content in guard

cells of intact leaves at the EoN and after 1 h and 3 h of illumination (*n* = 110 ± sem). Statistical significances determined by unpaired Student's *t* tests: # denotes $p < 0.05$ mutants versus WT at the indicated time point; * denotes $p < 0.05$ for the indicated comparisons; n.s. = not significant for the indicated comparison. (C) Representative confocal images of propidium iodide-stained guard cells of intact leaves. Scale bar = 10 µm. See also Figure S1.

Figure 3. Stomatal function and biomass production in response to light are impaired in *amy3bam1* mutants. (A) Stomatal aperture in WT and *amy3bam1* intact leaves during the light phase, determined by light microscopy and digital image processing (*n* = 100 ± sem). Statistical significances determined by unpaired Student's *t* tests: * denotes $p < 0.01$ for the indicated comparison. (B, C) Single leaf measurement of WT and *amy3bam1* stomatal conductance g_s (B) and internal CO_2 concentration (C) in 150 µmol m^{-2} s^{-1} light intensity ($n \geq 5$ ± sem). (D) Fresh weight of WT and *amy3bam1* rosettes of 4-week-old plants grown at different light intensities (*n* = 5 ± sem). Statistical significances determined by unpaired Student's *t* tests: * denotes $p < 0.01$ for the indicated comparison; n.s. = not significant for the indicated comparison. See also Figure S2.

Figure 4. Blue light signaling and proton pumping activate guard cell starch degradation during stomatal opening. (A) Starch content in guard cells of WT plants illuminated with blue light (75 µmol m^{-2} sec^{-1}) or red light (300 µmol m^{-2} sec^{-1}) after the end of the dark period (*n* = 110 ± sem). Unpaired Student's *t* test determined statistical significance between EoN and 1 h and 3 h light (# $p < 0.01$). (B) Guard cell starch content of WT and *phot1phot2* intact leaves during the first hour of the light period (*n* = 110 ± sem). Statistical significances determined by

unpaired Student's *t* tests: * denotes $p < 0.001$ for the indicated comparison; # denotes $p < 0.001$ between EoN and 45 min and 60 min of light for the *phot1phot2* mutant. (C) Starch accumulation in guard cells of intact leaves at the EoN and after 1 h and 3 h of illumination ($n = 110 \pm$ sem). Unpaired Student's *t* test determined statistical significance between the indicated comparisons (* $p < 0.01$; ** $p < 0.001$; n.s. not significant). (D) Guard cell starch content in WT epidermal peels treated for 1 h and 3 h with or without 2.5 µM tautomycin in opening buffer containing 50 mM KCl. 1 h dark-adapted peels were illuminated with 10 µmol m^{-2} sec^{-1} blue light superimposed on 50 µmol m^{-2} sec^{-1} red light ($n = 100 \pm$ sem). Unpaired Student's *t* test determined statistical significance between the indicated comparisons (* $p < 0.001$; n.s. not significant). (E and F) Stomatal aperture (E) and guard cell starch content (F) in epidermal peels treated for 1 h and 3 h with or without 10 µM fusicoccin (Fc) in opening buffer containing 50 mM KCl. 1 h dark-adapted peels were illuminated with 10 µmol m^{-2} sec^{-1} blue light superimposed on 50 µmol m^{-2} sec^{-1} red light ($n = 80 \pm$ sem). Unpaired Student's *t* test determined statistical significance between the indicated comparisons (* $p < 0.001$; n.s. not significant). See also Figure S3.