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Abscisic acid and other plant hormones: Methods to visualize distribution and signaling

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Abstract

The exploration of plant behavior on a cellular scale in a minimal invasive manner is key to understanding plant adaptations to their environment. Plant hormones regulate multiple aspects of growth and development and mediate environmental responses to ensure a successful life cycle. To monitor the dynamics of plant hormone actions in intact tissue, we need qualitative and quantitative tools with high temporal and spatial resolution. Here, we describe a set of biological instruments (reporters) for the analysis of the distribution and signaling of various plant hormones. Furthermore, we provide examples of their utility for gaining novel insights into plant hormone action with a deeper focus on the drought hormone abscisic acid.

Keywords

abscisic acid; fluorescent reporter; in vivo visualization; plant hormone

Introduction

Plant hormones are mobile chemical compounds that mediate growth and developmental processes on a global and also on a cellular scale to enable plants a successful life cycle under diverse environmental conditions. Chemical compounds designated as plant hormones are auxins (IAA), abscisic acid (ABA), brassinosteroids (BR), cytokinins (CK), ethylene (ET), gibberellins (GA), jasmonates (JA), karrikins (KAR), salicylic acid (SA), and strigolactones (SL). Plant hormones are involved in the promotion (ET, GA, KAR, SL) or inhibition (ABA) of seed germination, the inhibition of root growth (ABA, IAA, ET, JA), apical dominance (IAA, CK, SL), flower and fruit development (IAA, ET, GA, JA), delay (CK) or promotion (ET) of senescence, abiotic (ABA, BR) and biotic (BR, JA, SA, ABA) interactions, gravitropic responses (IAA, BR), and cell division and/or expansion (IAA, BR, CK, GA). This short summary does not present the whole picture of plant hormone functions. For more recent and specific overviews of plant hormones, we refer the reader to

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other publications [1–16]. Plant hormone signaling pathways communicate with each other on several levels. These include transcriptional responses, interactions of core transcription factors that are regulated by plant hormones, and regulation of hormone metabolism and transport [17–21].

Plant hormones are chemically diverse, and biosynthesis, degradation, or inactivation often involve multiple cellular compartments. For example, precursors or building blocks for the synthesis of IAA, ABA, GA, JA, and SL originate from plastids [11, 22–24]. SA and the most biologically active CKs are fully synthesized in plastids [25, 26]. An overview of plant hormone metabolic pathways and genes involved in these processes is provided from the RIKEN Plant Hormone Research Network (http://hormones.psc.riken.jp/pathway_hormones.shtml).

The distribution of plant hormones is not only dependent on the expression of the respective biosynthetic and metabolizing genes but also on the expression and activity of plant hormone transporters. Plant hormone transport is facilitated through various transport proteins. However, hormone transporters often belong to the ATP-binding cassette (ABC)-type transporter or Nitrate Transporter 1/Peptide Transporter Family (NPF) [27–29]. The hormone best understood in terms of its transport is IAA [30]. IAA uptake is facilitated through proton-coupled transporters of the AUX1/LAX family and through NRT1.1/NPF6.3 [31, 32]. Auxin efflux is regulated by PIN-type and ABC-type (ABCB1, 4, 19) transporters (summarized in [30]). ABA transport is also relatively well understood [33]. ABA is imported by ABCG40 and AIT/NPF-type transporters (AIT1/NPF4.6, AIT3/NPF4.1) [34, 35] and exported by ABCG25 and DTX50 [36, 37]. However, just recently it became evident that NPF4.1/AIT3 not only transports ABA, but also GA and JA-Ile [29, 35]. Other recent reports identified ABC-type transporters for CK (ABCG14) [38, 39] and SL (*Ph*PDR1) [40]. In the case of brassinosteroids, it is believed that they are not transported over long distances, and rather affect long-distance signaling through modulation of IAA transport [41].

There are certain similarities in hormone perception and signal transduction mechanisms. The mechanism of IAA, GA, and JA signal transduction is based on the hormone-dependent SKP1, Cullin, F-box (SCF)-complex ubiquitin ligase (E3)-mediated ubiquitination and 26S proteasomal degradation of transcriptional repressors [42, 43]. There are indications that KAR, SA, and SL also might signal through such mechanisms [13, 14, 44]. Core components of the ABA and ET signaling pathway are also subject to proteasomal degradation [42]. Other hormones signal through phosphorylation/ de-phosphorylation cascades (ABA, BR, CK, ET) [8, 43].

The coordination of plant hormone metabolism and transport and the regulation of core signaling component expression are crucial for maintaining tissue and cell-type specific hormone concentrations and signaling efficiency to facilitate adequate growth and developmental responses. Here, we describe recent advances in monitoring plant hormone distribution and signaling, and present an in-depth discussion of ABA.

Methods for the quantification and visualization of plant hormone distribution and signaling

Several methods can be used to detect and quantify plant hormones as well as plant hormone signaling. These methods encompass classical assays that require hormone extraction from ground tissue as well as the use of genetically encoded hormone reporters that enable in vivo hormone analyses. In the following, we will discuss current methods to analyze plant hormones and finish with a detailed discussion on methods to visualize ABA distribution and signaling.

Classical methods for the quantification of plant hormones

Quantitative measurements of plant hormones are often performed by liquid- and gas-chromatography coupled to mass spectrometry [45–47]. Other techniques are biological assays or immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) (summarized in [48, 49]). Although such methods have a high sensitivity for detecting hormones, their ability to resolve spatial and cell-type specific differences in hormone concentration is relatively low [45, 50]. Also, such methods most likely require hormone extraction from ground tissue. Therefore, these methods are not preferred for in vivo detection and measurements of plant hormones.

Fluorescently labeled plant hormones

Recently, it has become possible to fluorescently label IAA, BL, GA, and JA [51–55]. Except of NBD-IAA [55], such compounds appeared to be biologically active. Uptake experiments of externally applied fluorescently labeled hormones revealed that NBD-IAA accumulated in the endoplasmatic reticulum (ER) [55]. GA-FL accumulated in the root endodermis and this distribution pattern was affected by ET signaling [53]. The fluorescent BR analog AFCS was used to visualize BR receptor-ligand complexes in plants and its cellular uptake followed an endosomal trafficking route to the vacuole [51]. Fluorescently labeled plant hormones are ideal tools for the investigations of hormone uptake and transport. However, these compounds can only be used for exogenous treatments and their cellular distribution reflects only the distribution of actively transported hormone pools.

Expression-based hormone reporters

Expression-based hormone reporters are natural occurring hormone-responsive promoters or synthetic promoters containing repeats of hormone-responsive elements that are enhanced by a minimal p35S promoter and fused to a reporter gene (Table 1A) [56]. It should be clarified that such constructs report hormone signaling events rather than hormone concentration, because the reporter gene expression requires the whole hormone signaling cascade. The most prominent example of an expression-based reporter is the synthetic IAA-responsive pDR5 promoter [60], which has now been improved in terms of hormone sensitivity (pDR5v2 [62]). In addition, expression-based reporters have also been generated for ABA, CK, ET, JA, and SA (Table 1A and references therein). To date, natural hormone-responsive promoters/genes can be identified, for example, through search in publicly available gene expression datasets using the Genevestigator gene search tool [69]. However,

synthetic hormone-responsive promoters might be more specific for a certain plant hormone or hormone-activated transcription factor (gene family).

Reporter genes used for fusion to the hormone-responsive promoter are β-glucuronidase (GUS), luciferases (Luc), or fluorescent proteins (FP). A comparison of these reporter genes for expression studies in plants is provided in [70]. Although the use of the reporter gene depends on experimental needs, GUS assays require histochemical or fluorometric methods [71] that do not allow for direct in vivo investigations. On the other hand, Luc requires luciferin as substrate [70] and FPs require a certain maturation time before becoming fluorescent [72, 73]. In comparison, GUS and FPs are preferred at cellular resolution, while Luc is preferred at tissue and whole plant resolution [70].

Expression-based hormone-responsive reporters are not only suitable for the analyses of hormone signaling but are also powerful tools for genetic screens [57, 61, 74].

Protein degradation-based hormone reporters

Protein degradation-based hormone reporters utilize the hormone-specific SCF-complex-mediated proteasomal degradation of transcriptional repressors or repressor domains that are fused to FP or Luc (Table 1B, summarized in [56]). Such reporters have been developed for IAA, GA, and JA (Table 1B and references therein). Protein degradation-based hormone reporters can be subdivided into classical reporters that have been initially used to study GA (GFP-RGA [77]) or JA (JAI3-GFP [78]) signaling, and more advanced synthetic reporters for IAA (DII-Venus [75]) and JA (Jas9-Venus [79]) that opened the field for protein degradation-based hormone reporter analyses.

Protein degradation-based hormone reporters have the potential to semi-quantitatively report hormone concentrations because they most likely directly bind to the respective hormone receptor in a hormone concentration-dependent manner. Note that the abundance of the reporter not only depends on the efficiency of the hormone-specific SCF-complex but also on the efficiency of the expression cassette (promoter and terminator) that drives the reporter expression in a certain tissue or cell-type. While, the kinetics of hormone reporter degradation provide an approximate measure for the respective hormone concentration in a certain tissue or cell-type [75, 79], their ability to report hormone concentration reduction depends on the timescale of the respective hormone response and on the rate of de novo reporter synthesis.

For quantitative measurements, ratiometric reporters are preferred because they can be normalized for expression levels and because they are more suitable for prolonged microscopic analyses [80]. Approaches for normalizing the protein degradation-based IAA reporter DII-Venus [75] led to the development of R2D2 [62] and L2min17-Luc [76]. For ratiometric analyses, R2D2 combines the IAA-degradable DII domain of IAA28 [75] fused to n3xVenus (DII-n3xVenus) with the non-degradable mDII fused to ntdTomato (mDII-ntdTomato) [62]. To minimize effects of differential expression levels, both constructs were expressed under control of the pRPS5A promoter and located together on a single T-DNA [62]. L2min17-Luc is a protein consisting of Renilla and Firefly Luc linked by the A2 peptide and a 13 amino acid peptide of the IAA-degradable IAA17 [76]. Upon expression,

the A2 peptide is cleaved, resulting in stoichiometric amounts of the non-degradable Renilla Luc and the IAA-degradable min17-Firelfy Luc [76]. Both Luc signals are then ratiometrically quantified.

Protein degradation-based hormone reporters enabled the mapping of IAA and JA distribution and signaling in certain tissues and cell-types and investigations of changes in hormone distribution and signaling in response to gravitropic changes and wounding [62, 75, 79, 81].

FRET-based hormone reporters

FRET-based hormone reporters have been developed so far only for ABA [82, 83]. They consist of a fluorescent protein FRET-pair, usually improved variants of CFP and YFP that are linked together by a hormone-responsive sensory module. Upon hormone binding, the sensory module changes its structural conformation, thereby affecting the distance and orientation of the FRET-pair and their FRET efficiency [56, 84]. The FRET-based ABA reporters bind directly to ABA and therefore instantaneously visualize ABA concentration changes rather than ABA signaling [82, 83]. Note that the amount of bound hormone is not quantified through FRET efficiency measurements, but through simple YFP/CFP emission ratio measurements, enabling an internal normalization. In the discussion that follows, the FRET-based ABA reporters ABACUS [82] and ABAleon [83] will be compared and discussed in greater detail.

ABACUS and ABAleon are FRET-based reporters for ABA

Both ABA reporters ABACUS [82] and ABAleon [83] use FRET (“energy transfer from an excited donor fluorescent protein – CFP variant – to an acceptor fluorescent protein – YFP variant”) [85, 86] as a measure for the ABA concentration. Based on the mechanism of ABA perception and signaling and on structural analyses [87–90], their sensory modules were designed of an ABA receptor (PYR1 or PYL1) that is fused via a linker to a domain of the protein phosphatase type 2C (PP2C) ABI1. The sensory module is then linked to and sandwiched by the fluorescent protein FRET-pair (Fig. 1). Although both FRET-based ABA reporters use the same principle, their features in terms of used FRET-cassette, sensory module, and linkers differ quite strikingly (Fig. 1). Both ABA reporters appear to be flipped relative to the other in terms of the orientation of the FRET-pair and the orientation of the sensory module.

The differences in both ABA reporters are also reflected in their biochemical properties (Table 2). While ABACUS versions exhibit a low ABA affinity and high dynamic range, ABAleon versions bind ABA with high affinity but respond only with a low dynamic range (Table 2) [82, 83]. Still, compared to the advanced FRET-based calcium reporter yellow cameleon YC3.6 that has a dynamic range of 5.6 [91], the first generation FRET-based ABA reporters exhibit a relatively low dynamic range. The design of ABA reporters with higher dynamic range should be considered for future reporter engineering. It also needs to be mentioned that upon ABA binding, ABACUS exhibits a higher energy transfer to the YFP variant, while ABAleon exhibits a lower energy transfer [82, 83].

"Dimeric" ABA receptors, including PYR1 and PYL1, exhibit ABA affinities of K_d ~50–100 μM, while "monomeric" ABA receptors bind ABA with K_d ~1 μM (summarized in [92]). Conversion of PYR1 to an enhanced "monomeric" state by introducing the H60P mutation resulted in a Kd for ABA of ~3 μM [92]. Consistently, a homologous mutation in PYL1 H87P led to the conversion of ABACUS1-80μ to the higher affinity ABACUS1-2μ (Table 2) [82]. Introduction of the PYR1 H60P mutation in the ABAleon version ABAleon2.11 resulted in a non-ABA-responsive reporter [83]. "Monomeric" ABA receptors have the potential to interact with PP2Cs in the absence of ABA [87, 88, 93] which could also be the case for the sensory module of ABAleon2.11. ABAleon-type ABA reporters have a K'd for ABA comparable to ABA affinities of PYR1/PYL/RCAR-PP2C complexes that were measured between 18 and 125 nM (Table 2 and summarized in [94]). This implies that the ABAleon sensory module reflects the natural ABA-binding ability of PYR1 in complex with ABI1.

Based on structural and biochemical information, lower affinity ABAleon-type ABA reporters have been generated of which ABAleon2.15 – containing the PYR1 H115A mutation (Table 2) – exhibited a strong stereospecificity for the natural ABA enantiomer (+)-ABA [83]. A homologous amino acid in PYL3 (H139) was also found to be important for ABA stereospecificity [95], suggesting that ABAleon can be utilized to study biochemical properties of ABA receptors.

ABACUS1-2μ and ABAleon affect ABA responses

One feature of both ABA reporters is that they in some way affect ABA signaling. When Arabidopsis plants overexpress ABA receptors, as positive regulators of ABA signaling, they appear more sensitive to ABA [83, 88, 93, 96]. In contrast, over-expression of PP2Cs, as negative regulators of ABA signaling, renders plants less sensitive to ABA [93, 97]. ABACUS1-2μ lines exhibit an increased ABA sensitivity in seed germination and root growth inhibition assays [82]. These data indicate that $PYL1_{H87P}$ encoded in the ABACUS1-2μ sensory module might affect ABA signaling.

ABAleon2.1 lines were less sensitive to ABA during seed germination and seedling growth [83], and exhibit an overall reduction in growth when compared to Col-0 wild-type plants (Fig. 2A and B; [83]). The reduced growth was particularly evident for the high expressing ABAleon2.1 line 3 compared to the low expressing line 10 when grown in soil at relative air humidity of ~40% (Fig. 2A). Interestingly, growth of ABAleon2.1 line 3 was comparable to a line over-expressing the PP2C ABI1 fused to YFP (*abi1*-3/YFP-ABI1 [97]) (Fig. 2A). Note that ABAleon2.1 plant growth in soil can be improved when grown at higher relative air humidity (e.g. 70%; Waadt et al., unpublished).

Previous analyses revealed that ABAleon2.1 lines were less sensitive to ABA-mediated growth inhibition when 10 μM ABA was applied externally to in vitro grown seedlings [83]. We performed similar (root) growth inhibition analyses and transferred 4-day-old Arabidopsis seedlings to 0.5 MS media supplemented with either 0 μM ABA (control) or 30 μM ABA and grew the seedlings for additional 5 days (Fig. 2B–D). Growth on media containing 30 μM ABA reduced the overall growth, measured as fresh weight, in all investigated lines (Fig. 2C). Compared to Col-0 wild-type and in line with their role in ABA

signaling, ABA-induced fresh weight reduction was less pronounced for *abi1-3*/YFP-ABI1 and increased for YFP-PYR1 lines. ABAleon2.1 lines were also less sensitive to ABA in this respect (Fig. 2C). In 0 μM ABA conditions, ABAleon2.1 lines exhibited a reduced root growth when compared to Col-0 wild-type, consistent with the overall reduced growth in soil (Fig. 2A, B, and D). In the presence of 30 μM ABA, root growth of ABAleon2.1 lines and *abi1-3*/ YFP-ABI1 was less inhibited compared to Col-0 wild-type (Fig. 2D). YFP-PYR1 roots barely grew in these conditions (Fig. 2B and D).

There are two hypotheses that could explain the growth and ABA-response phenotypes of ABAleon2.1 plants: (i) due to the high ABA affinity, ABAleon2.1 might sequester physiologically relevant ABA pools and therefore suppress ABA responses [83]. In line with this hypothesis, it was found that the degree of ABA signaling suppression by ABAleon2.1 was dependent on its expression levels (Fig. 2D; [83]); (ii) the ABI1 domain encoded in the ABAleon sensory module might affect ABA signaling, even though it carries a phosphatase inactive mutation [83]. In line with this hypothesis, various transgenic lines of low affinity ABAleon versions also exhibited a reduced growth in soil (Waadt et al., unpublished). In the future, it will be important to develop FRET-based ABA reporters with reduced impact on ABA signaling.

Applications of ABACUS and ABAleon

ABACUS and ABAleon can be utilized for various aspects of ABA research. As both reporters instantaneously respond to ABA but also to the ABA mimic Pyrabactin [82] (Waadt et al., unpublished), they can be used to screen for other ABA analogs or compounds that suppress ABA binding by PYR1/PYL1 or interfere with the ABA-induced interaction of PYR1 with ABI1. The identification of ABA analogs and the engineering of ABA receptors is important for agrochemical approaches to improve water use efficiency [88, 96, 98].

ABACUS is able to detect ABA uptake into yeast cells after co-expression with the ABA importing transporter AIT1 [82]. Screening cDNA or transporter libraries in heterologous systems provides a powerful approach for the identification of novel ABA transporters [35]. In this respect, ABACUS and ABAleon could be used as another experimental readout to identify and characterize ABA transporters.

In Arabidopsis, ABACUS and ABAleon have been initially used to study ABA uptake. A 15 minutes pulse of ABA application induced an emission ratio increase of ABACUS, which declined after the ABA supply stopped [82]. These data indicated that ABA binds to ABACUS reversibly and that ABA is either quickly metabolized or exported from the cytoplasm. Studies with ABAleon2.1 seedlings revealed that ABA uptake was faster in the root elongation – compared to the root maturation zone. ABA was transported from shoot to root and hypocotyl to shoot, but not, in live cells, from root to shoot under the imposed experimental conditions [83]. The ABA transport rate within the hypocotyl was calculated to be ~16 μm/minute. In Arabidopsis, shoot-to-root ABA transport is consistent with the observations that in response to drought the shoot was the main source for rapid de novo ABA synthesis, which was then required to be translocated to the roots [58, 99].

Rapid FRET-based hormone reporters can be used to uncover time-resolved localized hormone concentration changes in response to diverse stimuli. For example, the time course of tissue and cell specific responses to salinity, osmotic stress and humidity changes have been investigated in initial studies [82, 83]. ABACUS and ABAleon detected salt stress (100–150 mM NaCl) induced [ABA] increases in leaves and guard cells [82, 83]. Interestingly, osmotically stressed (300 mM sorbitol) excised leaves did not accumulate ABA in guard cells [83]. In roots, both salt- and osmotic-stress triggered elevations in [ABA] [83].

When ABAleon2.1 plants were subjected to a drop in relative air humidity guard cell [ABA] elevated within 15 minutes [83]. The mechanism of ABA involvement in low humidity responses is still a matter of debate. Some studies suggest that a humidity drop causes a rapid rise in guard cell [ABA] (e.g. within 1–4 minutes), which would be required if ABA mediates the rapid humidity response. Other studies suggest that the humidity response does not require a rapid rise in guard cell [ABA] [100–104]. Stomatal conductance started decreasing within 5 minutes after a relative humidity drop [103, 105]. Knowledge about the exact time frame for reduced humidity-induced ABA synthesis would be important to evaluate the importance of ABA in reduced humidity-mediated stomatal closure. One model has been discussed in which the basal ABA levels may aid in the low humidity response without a requirement for rapid [ABA] changes in guard cells [103]. It has to be noted that in guard cells, the ABA sensitivity of ABAleon2.1 is quite low. Due to the high ABA affinity of ABAleon2.1 and the high basal [ABA] in guard cells [83], ABAleon2.1 appears often saturated. In the future, direct ABA reporters with an increased dynamic range and reduced ABA affinity (e.g. ABAleon2.15 [83] and ABACUS1-2m [82]) should allow direct analyses of the dynamics of humidity-induced [ABA] changes in guard cells.

Many other biological questions can be investigated with the newly emerging rapid FRET-based ABA reporters. For example, in present models [CO_2] changes that control stomatal movements suggest that the ABA and CO_2 signaling pathways converge downstream of ABA synthesis and perception [106]. Rapidly responding ABA FRET-based reporters will allow future direct studies into this presently untested hypothesis and question whether [CO_2] changes cause rapid changes in [ABA] in guard cells.

Distribution of ABA and ABA signaling in Arabidopsis

Because the FRET-based ABA reporters ABACUS and ABAleon are ratiometric and normalized, they can be used to visualize the distribution of ABA in intact and live plants. ABA distribution maps on seedlings have been generated using the highly expressing ABAleon2.1 line 3 [83]. These analyses revealed that [ABA] maxima exist in the root-tip, in the hypocotyl–root junction, and in guard cells. Furthermore, an [ABA] gradient appeared to exist in roots. ABAleon2.1 reported high [ABA] in the root base, with decreasing [ABA] toward the root-elongation zone that appeared to be a zone of minimal [ABA]. From there [ABA] increased towards the roottip [83]. These data are consistent with an ABA distribution map generated using the independent ABAleon2.1 line 10 (Fig. 3A). The [ABA] maxima reported by ABAleon2.1 are reflected in the expression pattern of *NCED* genes

involved in ABA biosynthesis [107, 108]. High [ABA] in guard cells is also consistent with their ability to autonomously synthesize ABA [102].

Differences in hormone concentrations do not necessarily reflect the signaling efficiency of a hormone in certain tissues or cell-types [75, 109]. For example, in Arabidopsis embryos of the heart stage [auxin] was high in the shoot apical meristem detected by the protein degradation-based reporter R2D2. However, the expression-based auxin signaling reporter DR5v2-n3GFP was not detected in the same cells [62]. These data indicate that hormone abundance not necessarily induces hormone signaling.

To compare ABA distribution and ABA signaling, ABAleon2.1 seedlings were investigated together with the expression-based reporter line pRAB18-GFP (Fig. 3). *RAB18* is a gene for which expression is induced by ABA [110, 111]. The pRAB18-GFP reporter has been initially established to screen for chemical compounds that affect ABA signaling [59]. Note that in Fig. 3, high [ABA] or signaling is represented in blue color and low [ABA] and signaling in red. Similar to the ABA distribution map generated by ABAleon2.1, pRAB18-GFP reported enhanced ABA signaling in guard cells (seen as blue dots on the cotyledons) and in the hypocotyl–root junction (Fig. 3). Compared to the high [ABA] in the root tip, ABA signaling was only moderate. Striking differences between both reporters were found in the root, where pRAB18-GFP expression was at the detection limit of the microscope and ABAleon2.1 reported comparably high [ABA], except in the root-elongation zone (Fig. 3A and B, left panels). External application of 50 μM ABA resulted in ABA uptake and increased ABA signaling (Fig. 3A and B, right panels). While [ABA] increased in the entire seedling after external ABA application, ABA-induced ABA signaling was more evident in the root and the hypocotyl–root junction. Note that pRAB18-GFP responds slower to externally applied ABA, as expression of the GFP reporter gene requires time for signaling, mRNA transcription, translation, and GFP maturation. The ABA signaling map reported by pRAB18-GFP was consistent with observations using other ABA-responsive expression-based reporters (pRD29B and pAtHB6, [58]). In the future, it will be interesting to re-evaluate the [ABA] map using improved next generation ABA reporters, and to elucidate the role of the hypocotyl–root junction in ABA-mediated responses and the reason for the apparent reduction in the endogenous ABA sensitivity of roots compared to guard cells.

pRAB18-GFP reports changes in ABA signaling in response to abiotic stresses

It is important to know how certain tissues respond to stresses that affect ABA signaling. pRD29A/B-Luc and pAtHB6-Luc reporters have been used to study cold, salt, and osmotic stress responses [57, 58, 112]. These analyses provided evidence for the involvement of ABA in abiotic stress responses and led to the identification of ABA synthesis genes [113, 114]. The pRAB18-GFP reporter has been used for a chemical genetic screening and to study salt stress responses in roots and humidity responses in guard cells [59, 115–117]. As pRAB18-GFP uses a fluorescent protein as reporter gene, this reporter might have a higher potential to resolve tissue and cell-specific differences in ABA signaling.

Five-day-old seedlings of the expression-based ABA signaling reporter pRAB18-GFP were incubated for 6 hours in control media and media containing 10 μM ABA, 100 mM NaCl (salt stress), or 300 mM sorbitol (osmotic stress) (Fig. 4). Analyses in hypocotyls (Fig. 4A) and two different zones of the root (Fig. 4B and C) indicated a strong reporter induction in response to 10mM ABA. In the hypocotyl and root-maturation zone, salt and osmotic stress induced pRAB18-GFP expression, but to a lesser extent compared to 10 μM ABA treatment (Fig. 4A – D). Also in these tissues, the osmotic stress response was approximately twofold higher compared to the salt stress response (Fig. 4B and D). The induction of ABA signaling in the root maturation zone in response to salt and osmotic stress was consistent with increased [ABA] measured using ABAleon2.1 [83]. In the early maturation zone, salt stress did not affect pRAB18-GFP expression, while osmotic stress responses were comparable with ABA responses (Fig. 4E and F). Note that in this region salt stress induced [ABA] increases [83], but not ABA signaling (Fig. 4E and F). The root elongation zone, which is located directly below the early maturation zone, appeared to be special in terms of ABA responses. This region exhibited the lowest [ABA] in the root (Fig. 3A), but also a weaker pRAB18-GFP induction in response to ABA and sorbitol when compared to the early maturation zone (Figs. 3 and 4E).

In summary, the pRAB18-GFP reporter represents a useful tool to study ABA signaling in response to abiotic stresses and reveals differences in signaling intensity and distribution between salt- and osmotically stressed seedlings.

Conclusions and outlook

Plant hormones mediate various growth and developmental processes dependent on the specific natural environment and growth stage of a plant. Recent advances in fluorescent labeling of plant hormones and the development of genetically encoded reporters have opened new possibilities for researchers to study hormone uptake, distribution, signaling patterns, and hormone-mediated protein degradation in living intact plants. However, such tools remain to be established for each hormone (see Table 1). The first generation of FRET-based ABA reporters ABACUS and ABAleon affect ABA signaling, but also allow insights into the cellular dynamics and movement of ABA through the plant in real time. Future reporter engineering has the potential for many improvements, including their dynamic range and spectral characteristics. Design of reporters with non-overlapping fluorescence emission would enable the monitoring of various aspects of hormone action or multiple hormones at the same time. Using such reporters, future studies could include investigations of hormone transport, environmental responses, but also the analysis of hormone crosstalk/interdependence. In summary, the use of genetically encoded reporters will advance our understanding about plant hormones and lead to deeper knowledge of how plants grow, develop, and how they face their daily challenges with the environment.

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Abbreviations

ABA	abscisic acid
ABC	ATP-binding cassette
ABI1	ABA insensitive 1
AFCS	alexafluor 647-castasterone
AIT	ABA-importing transporter
ARR5	Arabidopsis response regulator 5
AtHB6	*Arabidopsis thaliana* homeobox protein 6
AUX1	auxin resistant 1
BR	brassinosteroid
CFP	cyan fluorescent protein
CK	cytokinin
DTX50	detoxification efflux carrier 50
ELISA	enzyme-linked immunosorbent assay
ET	ethylene
FP	fluorescent protein
FRET	Förster resonance energy transfer
GA	gibberellin
GFP	green fluorescent protein
GUS	β-glucuronidase
IAA	auxin
IAA17	IAA inducible 17
IAA28	IAA inducible 28
JA	jasmonate
JAI3	jasmonate insensitive 3
KAR	karrikin
K'_d	apparent affinity
LAX	like AUX1
LUC	luciferase
NCED	9-cis-epoxycarotenoid dioxygenase
NPF	nitrate transporter 1/peptide transporter family
obs1	optimal binding sequence for TEIL

p35S	cauliflower mosaic virus 35S promoter
PDR1	pleiotropic drug resistance 1
PP2C	protein phosphatase type 2C
PYL1	pyrabactin resistance 1-like
PYR1	pyrabactin resistance 1
RAB18	responsive to ABA 18
RCAR	regulatory component of ABA receptor
RD29A/B	responsive to dessication 29 A/B
RGA	repressor of GA
RPS5A	ribosomal protein 5A
SA	salicylic acid
SCF	SKP1, cullin, F-box-complex
SKP1	S-phase kinase associated protein 1
SL	strigolactone
TCS	two component signaling sensor
T-DNA	transfer-DNA
TEIL	tobacco EIN3-like
YC3.6	yellow cameleon 3.6
YFP	yellow fluorescent protein

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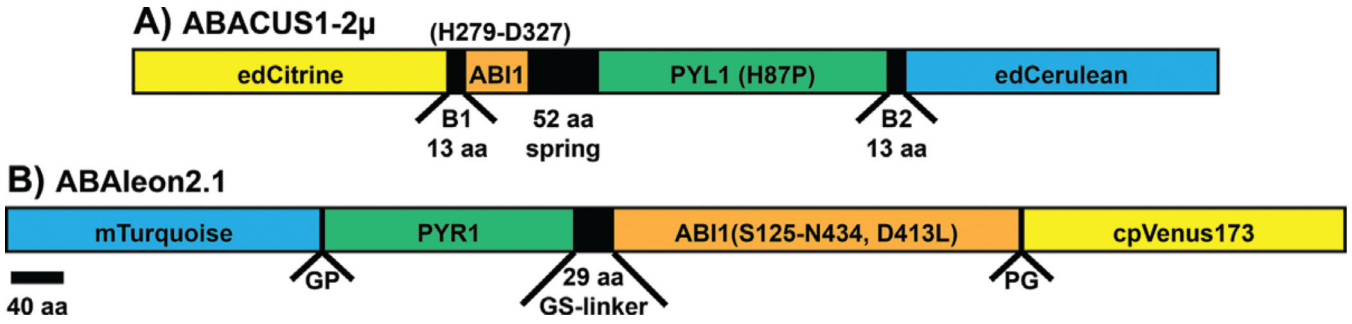

Figure 1.

Schematic presentation of the FRET-based ABA reporters ABACUS1-2µ (**A**) and ABAleon2.1 (**B**). As FRET donor and acceptor pair, ABACUS1-2µ uses the enhanced dimerization CFP- and YFP-variants edCerulean and edCitrine. ABAleon2.1 encompasses mTurquoise and Venus circularly permutated at amino acid 173 (cpVenus173) as FRET-pair. The N- and C-terminal orientation of the respective fluorescent protein is interchanged in both reporters. The sensory modules consist of a full length ABA receptor protein (PYL1 with the H87P mutation in ABACUS1-2µ and PYR1 in ABAleon2.1) fused to a fragment of the type 2C protein phosphatase (PP2C) ABI1. ABACUS1-2µ harbors 49 amino acids of the ABI1 phosphatase domain (amino acids H279-D327), while ABAleon2.1 contains the complete phosphatase domain (amino acids S125-N434), but with a D413L mutation that abolishes phosphatase activity. The orientation between PYR1/PYL1 and the ABI1 fragment is interchanged in both reporters. PYR1/PYL1 and ABI1 are linked through a 52 amino acid spring linker in ABACUS1-2 µ, and through a flexible 29 amino acid glycine-serine-(GS)-linker in ABAleon2.1. ABACUS1-2µ was generated using the gateway-cloning strategy, therefore the sensory module is linked to the fluorescent protein pair via 13 amino acid attB1 and attB2 sites. ABAleon2.1 was generated using classical cloning and linked to the fluorescent proteins through GP and PG linkers encoded in the *ApaI* and *XmaI* restriction sites. Informations about both reporters are derived from [82, 83].

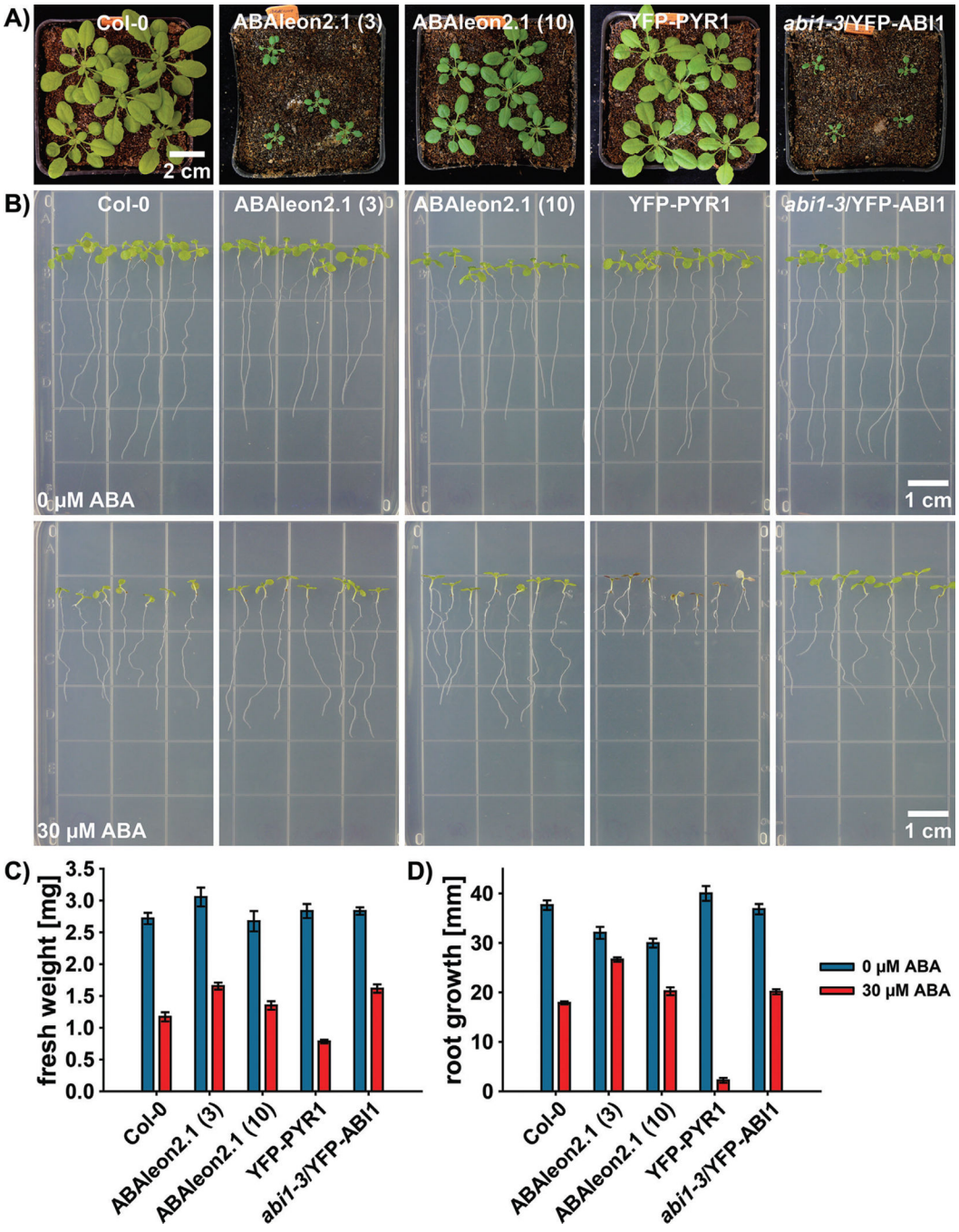

Figure 2.

ABAleon2.1 plants exhibit a growth phenotype and a reduced sensitivity to ABA. **A**: Twenty-five-day-old plants grown in parallel in a growth chamber at a relative humidity of ~40%. **B**: Four-day-old seedlings were transferred to 0.5 MS agar plates supplemented with 0 µM ABA (top row) or 30 µM ABA (bottom row) and grown for 5 additional days. **C** and **D**: Quantification of growth parameters (**C**) fresh weight and (**D**) root growth of seedlings presented in (**B**) 5 days after transfer to media supplemented with 0 µM ABA (blue bars) or

30 μM ABA (red bars). Data represent means ±SEM of $n = 5$ experiments with seven seedlings per experiment. Plants were grown and analyzed as described in [83].

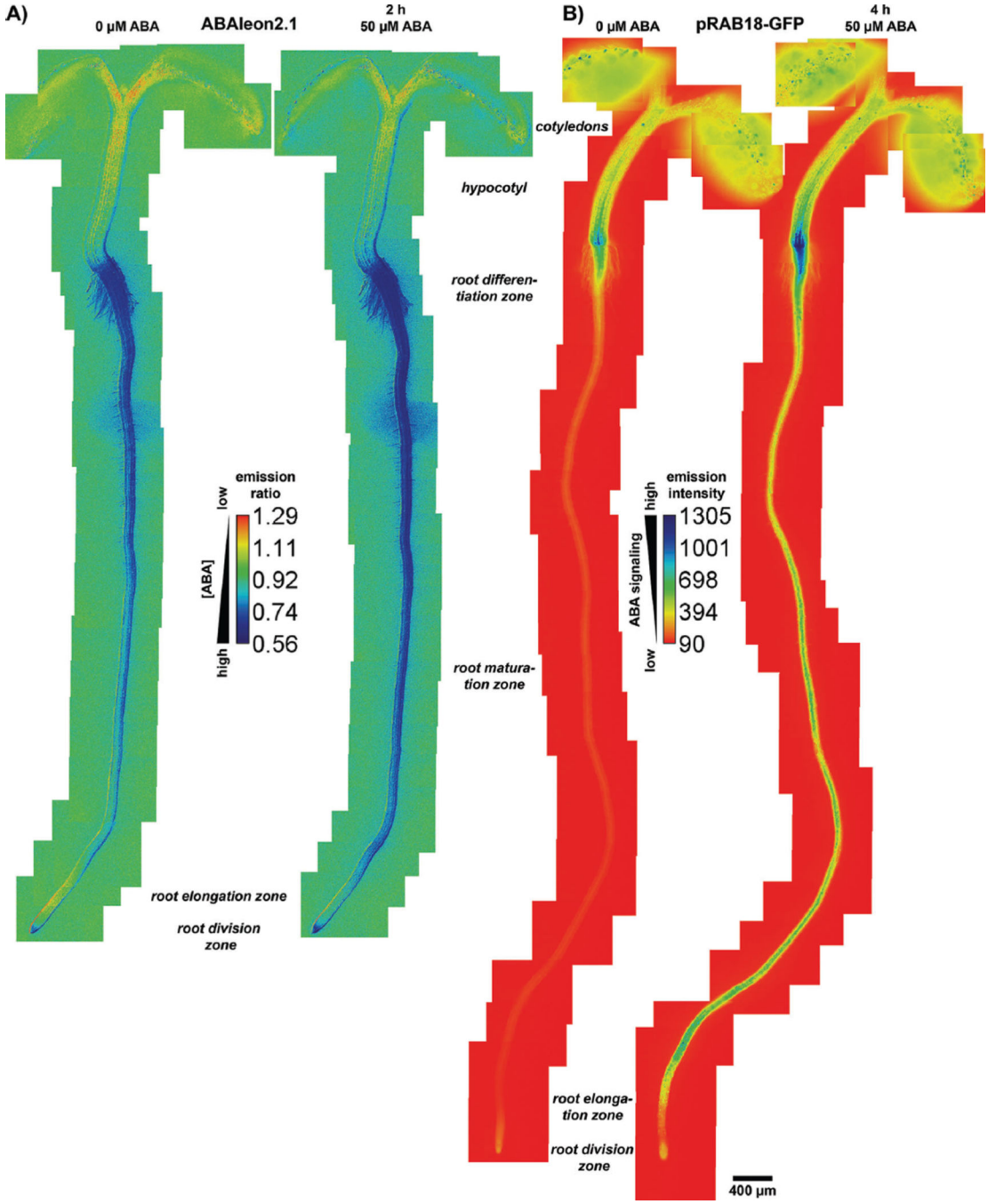

Figure 3.

Distribution of (**A**) [ABA] and (**B**) ABA signaling. Five-day-old seedlings of (**A**) ABAleon2.1 line 10 and (**B**) pRAB18-GFP were imaged before (left panel) or 2 hours (ABAleon2.1) or 4 hours (pRAB18-GFP) after application of 50 µM ABA (right panel). Shown are manually assembled emission ratio (ABAleon2.1 – reports [ABA]) or background subtracted fluorescence emission (pRAB18-GFP – reports ABA signaling) images calibrated to the respective calibration bar. Blue color indicates high [ABA] or signaling and red indicates low [ABA] or signaling. Background colors are according to the

respective calibration bars [values are ~1 in (**A**) and 0 in (**B**)]. Plants were grown, imaged, and analyzed as described in [83], except that imaging of pRAB18-GFP was conducted using a 71012pH sensitive GFP filter set (CHROMA Technology Corp.).

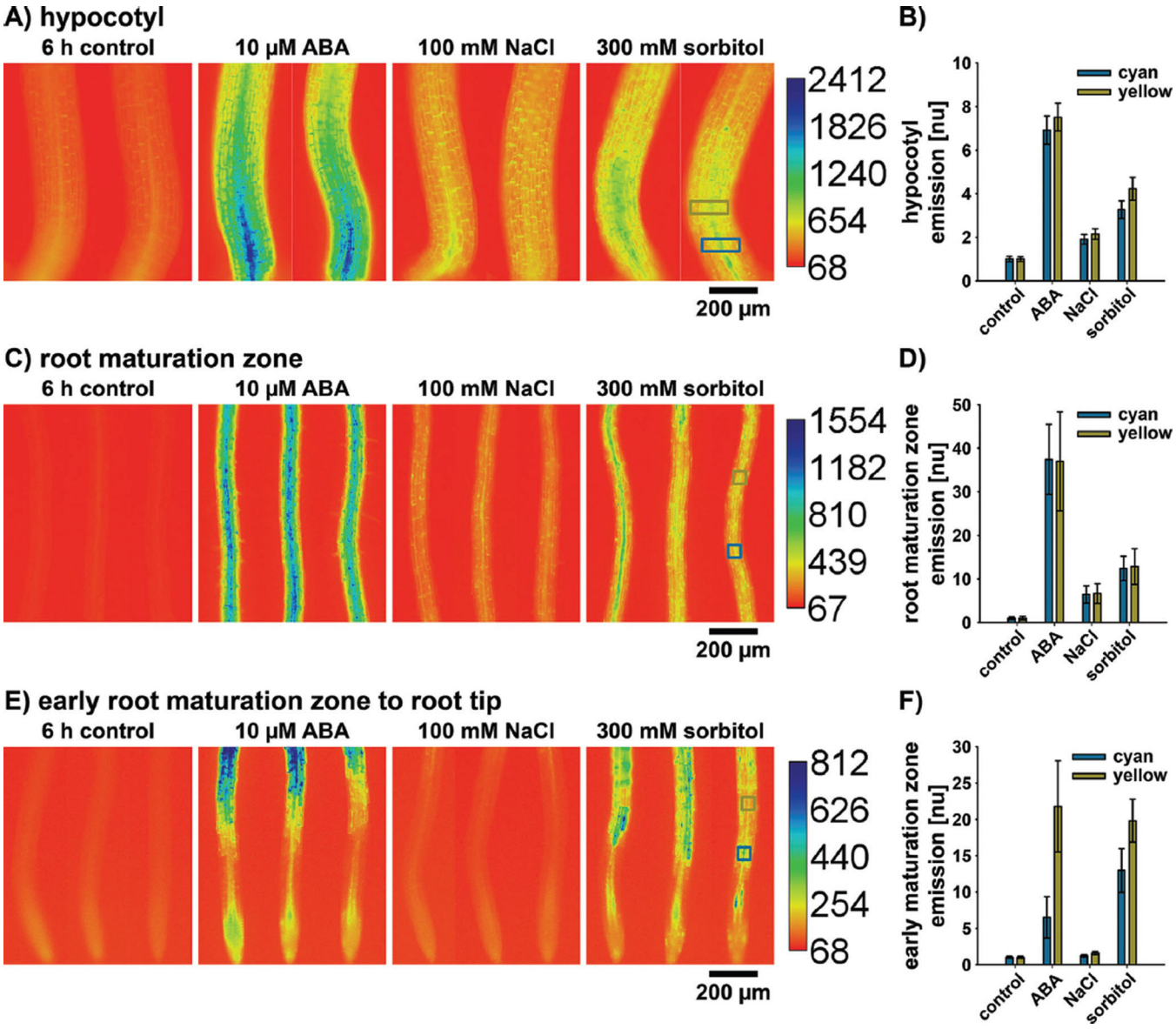

Figure 4.

ABA signaling in response to salt and osmotic stress. Fluorescence emission of the expression-based ABA signaling reporter pRAB18-GFP in (**A** and **B**) the hypocotyl, (**C** and **D**) the root maturation zone, and (**E** and **F**) the early maturation zone, elongation zone, and division zone in response to 6 hours control, 10 µM ABA, 100mM NaCl (salt stress), and 300 µM sorbitol (osmotic stress) treatments. Images were calibrated according to the adjacent calibration bar. **B, D**, and **F**: Background subtracted fluorescence emission values measured from two color-coded regions depicted in the right images of (**A, C**, and **E**) and normalized to the control. Data represent means ± SEM of $n = 8$ seedlings. The indicated color-coded regions are representative for regions analyzed in all acquired images with identical positions on the *Y*-axis.

Table 1

List of hormone-responsive reporters categorized by (A) expression-based reporters, (B) protein degradation-based reporters, and (C) FRET-based reporters with indicated

Hormone	Promoter	Reference
(A) Expression-based reporters		
Abscisic acid	pRD29A/B	[57]
Abscisic acid	pAtHB6	[58]
Abscisic acid	pRAB18	[59]
Auxin	pDR5	[60]
Auxin	pBA	[61]
Auxin	pDR5v2	[62]
Cytokinins	pARR5	[63]
Cytokinins	pTCS	[64]
Cytokinins	pTCSn	[65]
Ethylene	pobs1	[66]
Jasmonates	p4D-47	[67]
Salicylic acid	p4xWT-46	[68]
Hormone	Reporter	Reference
(B) Protein degradation-based reporters		
Auxin	DII-Venus	[75]
Auxin	L2min17-Luc	[76]
Auxin	R2D2	[62]
Gibberellins	GFP-RGA	[77]
Jasmonates	JAI3-GFP	[78]
Jasmonates	Jas9-Venus	[79]
Hormone	Reporter	Reference
(C) FRET-based reporters		
Abscisic acid	ABACUS	[82]
Abscisic acid	ABAleon	[83]

Table 2

Biochemical properties of FRET-based ABA reporters

ABA reporter	ABA receptor	K'_d [μM]	In vitro DR
ABACUS1-2 μ	PYL1 H87P	2	1.6
ABACUS1-80 μ	PYL1	80	2.6
ABAleon2.1	PYR1	0.1	1.1
ABAleon2.15	PYR1 H115A	0.5	1.1

Indicated are the reporter names, the ABA receptor incorporated in the sensory module, their apparent ABA affinity (K'_d) and in vitro dynamic range (DR) calculated from ABA-bound emission ratio/ ABA-unbound emission ratio.