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Spotlight

Plant Osmotic Stress Signaling: MAPKKKs Meet SnRK2s

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Osmotic stress signaling in higher plants is crucial to cope with abiotic stress. RAF-like MAPKKKs are activated by hyperosmotic stress and activate downstream ABA-unresponsive and ABA-activated SnRK2s, integrating early osmotic stress and ABA signaling cascades. The connection of B2/B3/B4 RAF-like MAPKKKs with SnRK2s is a new paradigm in signal transduction.

Plant Productivity Is Strongly Limited by Drought and High Salinity

Many environmental challenges (drought, salinity, freezing, high irradiation) can lead to water deficit [1]. Plants have sophisticated signaling cascades to match transpirational water loss and water absorption, and stress perception is crucial for plant coordinated response [1]. Water deficit generates osmotic stress (OS), which is likely perceived by the cell as turgor loss and triggers signaling cascades for osmotic adjustment (Figure 1). Convergent discoveries have shed light on the signaling mechanisms that mediate OS signaling in higher plants [2–6]. The osmosensors that perceive changes in turgor pressure remain

to be identified; however, the main downstream components (subfamily I and III SnRK2s) were well known and upstream activating kinases (UAKs) of SnRK2s have been recently discovered [2–6].

Adapting to water deficit was crucial for the colonization of land and required the key molecule ABA. ABA signal transduction is well established and involves the PYR/PYL/RCAR ABA receptors, which, upon ABA perception, relieve repression of sucrose nonfermenting1-related protein kinases2 (SnRK2s) subclass III by clade A protein phosphatases type 2C (PP2Cs). Thus, *Arabidopsis thaliana* subclass III SnRK2.2/3/6 are ABA-dependent for their activation and require PP2C inhibition by ABA and ABA receptors. Increase of ABA levels by *de novo* biosynthesis, transport, uptake in target tissues, or release from plant reservoirs requires some time. However, plants also show a fast response (within minutes) to OS, which is ABA-independent. This is mediated by ABA-unresponsive subclass I SnRK2s (i.e., SnRK2.1/4/5/9/10 in *Arabidopsis*) as well as subclass III SnRK2s [7]. As a result, genetic analyses have revealed redundancy among subclass I and III SnRK2s in the OS response [8]. Since OS-mediated activation of subclass III SnRK2s is ABA-independent, a signaling pathway was missing to explain their early activation as well as that of subclass I SnRK2s. Moreover, how are integrated hyperosmotic stress and ABA upstream of SnRK2s?

The initial answer to these questions was provided by studies in the moss *Physcomitrella patens* [9]. Core components of ABA signaling are present in *P. patens*, which are therefore conserved from bryophytes to angiosperms. A moss transgenic line lacking the two group A PP2Cs [ppabi1 a/b double mutant (see Glossary)] showed partial constitutive activation of ABA-activated SnRK2s, whereas ABA treatment led to enhanced activation of the ABA-activated SnRK2s compared

Glossary

112458 pyl sextuple mutant: impaired in the PYR1, PYL1, PYL2, PYL4, PYL5, and PYL8 ABA receptors.

abi1-2/abi2-2/pp2ca-1 triple mutant: loss-of-function mutations in three clade A PP2Cs of *A. thaliana*.

abi1^{G180D}, abi2^{G188D}, and hab1^{G246D} dominant mutants: encode PP2Cs that are refractory to inhibition by ABA and ABA receptors.

amiRNA_m3k: artificial microRNA line that impairs expression of 5 B3-MAPKKKs.

hab1-1/abi1-2/abi2-2 triple mutant: loss-of-function mutations in three clade A PP2Cs of *A. thaliana*.

MAPK: mitogen-activated protein kinase.

MAPKK: mitogen-activated protein kinase kinase.

MAPKKK: mitogen-activated protein kinase kinase kinase.

OK-quatdec mutant: impaired in 4 B2-, 3 B3-, and 7 B4-MAPKKKs, corresponding to RAF7/8/9/10, RAF3/4/5, and RAF16/18/20/24/35/40/42, respectively.

ppabi1 a/b double mutant: impaired in two clade A PP2Cs of *P. patens*.

raf2/edr1 raf4 raf5 raf10 raf11 pentuple mutant: impaired in 2 B2- and 3B3-MAPKKKs, also termed as OK¹⁰⁰-quin.

raf3/m3kδ1 raf4/m3kδ7 raf5/m3kδ6 triple mutant: impaired in 3 B3-MAPKKKs.

raf16 raf18 raf20 raf24 raf35 raf40/hcr1 raf42 septuple mutant: impaired in 7 B4-MAPKKKs, also termed as OK¹³⁰-weak (residual activity of raf24 and raf42) or OK¹³⁰-null (null alleles).

snrk2 decuple mutant: impaired in SnRK2.1/2/3/4/5/6/7/8/9/10.

snrk2.2/3/6 triple mutant: impaired in the subfamily III SnRK2.2/2.3/2.6.

Subfamily I SnRK2s: *Arabidopsis* SnRK2.1/4/5/9/10, ABA-unresponsive, OS-activated.

with wild type (wt). This suggested the presence of an unidentified positive mechanism for SnRK2 activation. Genetic studies led to the identification of the AR7 mutant, which lacks most ABA responses of the moss and shows little SnRK2 kinase activity after ABA or OS exposure. The identification of the gene mutated in AR7 led to the discovery of a protein kinase designated ARK (ABA and abiotic stress-responsive RAF-like kinase), which is encoded by a single gene and belongs to B3 subgroup of RAF-like MAPKKKs. Further biochemical characterization of ARK revealed the missing link that integrates ABA and hyperosmotic stress signaling upstream of SnRK2 [9].

RAF-Like MAPKKs Meet SnRK2s Instead of MAPKKs

The original concept of MAPKKK (i.e., the upstream component of a sequential cascade of activating kinases where a **MAPK** is activated by a MAPK kinase, which in turn is activated by a **MAPKK** kinase) has not been biochemically proved for most plant MAPKKKKs. This family of kinases has greatly expanded in angiosperms and, for example, *A. thaliana* contains 21 MEKK-like and 48 RAF-like MAPKKKKs [10]. In arabidopsis, group A comprises MAPKKKKs whose kinase domains have significant similarity to MEKK/STE11, whereas groups B (divided into B1 to B4 subgroups) and C are termed RAF-like kinases. Therefore, although plant RAFs are presumed to be MAPKKKKs by phylogenetic analyses based on their protein kinase catalytic domain, it is possible that biochemically they behave differently from mammalian RAFs.

Arabidopsis B3-MAPKKK genes, At1g73660/RAF5 and At4g24480/RAF6, were able to complement ABA insensitivity of AR7 mutant [9], which does not necessarily reflect a similar role in angiosperms; however, subsequent discoveries have established a functional link of B3-MAPKKKKs, as well as B2- and B4-MAPKKKKs, with SnRK2s in arabidopsis [2–6]. Given that B2/B3/B4 subgroups comprise 19 genes overall (Figure 1B), high order mutants were required to genetically support their role in the activation of SnRK2s. A redundancy-circumventing genetic screen based on artificial microRNA (amiRNA)-expressing lines that target gene families led to the isolation of MAPKKK amiRNA mutants able to germinate in the presence of 2 μ M ABA [2]. Three B3-MAPKKKKs, At5g11850/RAF3/M3K δ 1, At1g73660/RAF5/SIS8/M3K δ 6, and At1g18160/RAF4/M3K δ 7, reactivated previously dephosphorylated SnRK2.6 [2]. The *raf3/m3k δ 1 raf4/m3k δ 7 raf5/m3k δ 6* triple mutant shows reduced sensitivity to ABA but,

Figure 1. Effect of Water Deficit and Subsequent Plant Response Mediated by Osmotic Stress (OS) and ABA-Dependent Signaling Pathways. (A) Movement of water depends on the water potential (Ψ_w) gradient across the membrane. The root must establish a gradient to enable water flow from the soil towards the root surface. In the left panel, it is shown that by decreasing the solute potential (Ψ_s) through accumulation of compatible solutes such as proline, osmotic adjustment can drive Ψ_w to values lower than soil Ψ_w . The pressure potential (Ψ_p , turgor is positive pressure and generates Ψ_p greater than 0 MPa) is compensated by lowering Ψ_s . Under severe OS and no osmotic adjustment, water will flow out of the cell until turgor is lost (right panel). Water uptake by roots matches water loss by transpiration through regulation of stomatal aperture by ABA. (B) Cladogram of arabidopsis B2, B3, and B4 RAF-like MAPKKKKs. RAF1/CTR1, RAF2/EDR1, RAF5/SIS8, and RAF40/HCR1 were previously identified by genetic screenings. (C) Model to illustrate osmosensing and ABA perception to trigger stress adaptation. Prior to ABA accumulation, OS is perceived through unknown osmosensors, which generates activation of B2, B3, and B4 RAF-like MAPKKKKs. ABA

(Figure legend continued at the bottom of the next page.)

surprisingly, stomatal conductance was reduced compared with wt [2]. This latter phenotype is opposite to that of ***snrk2.2/3/6 triple mutant***, which shows higher stomatal aperture than wt. Finally, *raf3 raf4 raf5* is able to germinate on 2 μ M ABA, but *snrk2.2/2.3/2.6 triple* or ***112458 pyl sextuple mutants*** are resistant even to 50 μ M ABA. The ***amiRNA_m3k*** line targets 5 B3-MAPKKs but still shows activation of SnRK2.2/2.3/2.6 in response to ABA [2].

Analysis of an ***snrk2 decuple mutant*** revealed the importance of SnRK2s in OS response [8]. To investigate early events (minutes) in OS signaling, Lin *et al.* measured kinase activation induced by mannitol treatment [3]. In addition to subfamily I and III SnRK2s, the authors found two groups of kinases of approximately 130 and 100 kDa that were specifically activated by OS and not by ABA, termed as OS-activated protein kinases (OKs) [3]. Quantitative phosphoproteomics and mutational analyses identified OKs as RAF-like kinases. The authors could identify OK¹⁰⁰ as members of the B2/B3-MAPKKs, whereas OK¹³⁰ correspond to B4-MAPKKs [3]. Inactivation of the seven B4-MAPKKs in the ***OK¹³⁰-null*** septuple mutant does not affect OS-induced activation of subfamily III but abolishes activation of **subfamily I SnRK2s**, which points out an important branching

point in the cascade [3]. Thus, RAF16/18/20/24/35/40/42 B4-MAPKKs are UAKs for ABA-unresponsive subfamily I SnRK2.1/4/5/9/10. This finding is coincident with Soma *et al.* [4], who identified RAF18/20/24 as playing a major role for activation of subclass I SnRK2s. To identify those MAPKKs that mediate OS-induced activation of SnRK2.2/3/6, Lin *et al.* [3] edited B2/B3-MAPKKs to generate the ***raf2/edr1 raf4 raf5 raf10 raf11 pentuple mutant***, termed OK¹⁰⁰-quin, which shows strong loss of mannitol-induced activation of SnRK2.2/3/6 and establishes another branching point of UAKs for OS-induced activation of subfamily III [2,3] (Figure 1C).

A genetic 'tour de force' enabled the generation of a mutant impaired in 14 RAFs, including 4 B2-, 3 B3-, and 7 B4-MAPKKs (***OK-quatdec mutant***). This mutant shows reduced sensitivity to 1 μ M ABA during seed germination/seedling establishment and enhanced water loss, indicating that RAFs are required for ABA-mediated SnRK2 activation [2,3]. However, this ABA-insensitive phenotype in germination is weak compared with *112458* [11] and *snrk2.2/3/6* lost more water than OK-quatdec [3]. This suggests that full activation of **subfamily III SnRK2s** strongly depends on clade A PP2C inhibition by PYL ABA receptors. What about a PP2C role in OS-triggered activation of SnRK2s? A definitive answer

will require further analysis, given that only some interactions of clade A PP2Cs and SnRK2.2/3/4/6/10 have been reported. However, the ***abi1^{G180D}, abi2^{G168D}, and hab1^{G246D} dominant mutants*** block ABA-induced activation of subfamily III SnRK2s but do not affect OS-induced activation of both subfamily I and III [3,7,12]. Moreover, ***hab1-1/abi1-2/abi2-2 triple mutant*** and ***abi1-2/abi2-2/pp2ca-1 triple mutant*** show enhanced OS-induced activation of subfamily III SnRK2s but not of subfamily I SnRK2s [3,12].

Future Avenues of Research

Biochemical studies should distinguish the phosphorylation-based mechanisms that mediate SnRK2 activation by ABA and OS [7]. It is possible that other RAF-dependent phosphosites, different from Ser171 and Ser175, might circumvent PP2C-mediated inhibition of SnRK2s after perception of OS [3]. Osmosensing is currently a major gap in plant biology; in contrast, osmosensors and their connections with MAPK signaling cascades have been extensively studied in yeast. Finally, phosphoproteomic analyses have revealed ABA- and OS-induced phosphosites as potential MAPK targets; therefore, it is worthy to investigate whether RAF-like MAPKKs actually function in the canonical MAPK module.

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accumulation is fast in guard cells, which are autonomous for ABA biosynthesis, whereas in other tissues some time is required for transport from vascular tissues and uptake (ABCG- and NPF-type transporters for ABA uptake are indicated; protonated form of ABA can diffuse through plasma membrane). Perception of OS in vascular tissue involved in ABA biosynthesis should lead to upregulation of *NCED3*, the rate-limiting enzyme for ABA biosynthesis. The ABA-glucosyl ester (ABA-GE), stored in vacuole or endoplasmic reticulum, is released in response to water deficit and represents another source of ABA. Arabidopsis subfamily I SnRK2s are activated by B4-MAPKKs, whereas subfamily III SnRK2s are activated by B2- and B3-MAPKKs. Full activation of arabidopsis SnRK2.2/3/6 requires ABA and ABA receptors to release PP2C inhibition. Subfamily I SnRK2s control mRNA population by promoting mRNA decapping and subsequent mRNA degradation. In parallel, transcriptional activation is mediated by subfamily III SnRK2s. In guard cells, subfamily III SnRK2s (particularly SnRK2.6/OST1) promote anion (A⁻) and potassium (K⁺) efflux, which induces water efflux and stomatal closure to reduce water loss by transpiration. Slow and quick anion channels (SLAC/QUAC) promote anion efflux. Aquaporins of the plasma membrane intrinsic protein (PIP) family increase the permeability of biological membranes to water and facilitate hydrogen peroxide (H₂O₂) entry into guard cells. In other cells and tissues (e.g., roots), stress adaptation involves osmotic adjustment to maintain water uptake and turgor. This figure was created using BioRender (<https://biorender.com>).

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Spotlight

Tracking the Origin and Evolution of Plant Metabolites

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Iridoids are monoterpenes that are produced by various plants as chemical defense molecules. Lichman *et al.* recently described the timeline of molecular events that underpin the re-emergence of iridoid biosynthesis in an

independent lineage of aromatic plants (catnip). This study represents a benchmark for studying enzyme and metabolite evolution in different clades across the tree of life.

Plant Metabolites

Understanding the origin and evolution of plant metabolites is fundamental to explaining the distribution of natural products among plant families. These metabolites are produced by plants as a response to abiotic and biotic stress, and also mediate inter- and intra-species communication. They are widely used by humans in medicine and agriculture. Mapping the presence and absence of plant metabolites across angiosperms has been undertaken for many years [1], but understanding the molecular mechanisms and evolution of these metabolites remains more challenging. To determine whether a specific chemical family evolved independently in different lineages or arose from the same ancestral pathway, the identification of genes and proteins involved in the biosynthesis of these natural products is an essential prerequisite. For example, in the biosynthesis of plant tropane alkaloids, the enzymes responsible for the tropinone-reduction step are thought to have risen independently across angiosperm species [2]. Conversely, the norcoclaurine synthase for benzylisoquinoline alkaloid biosynthesis in angiosperms is suggested to be the result of monophyletic evolution before the emergence of eudicots [3]. Deciphering the evolution of terpene synthase (TS) enzymes across plant lineages is challenging because terpenes are present in the earliest lineages of land plants that colonized terrestrial habitats [480–430 million years ago (Mya)] [4]. The extensive superfamily of TS enzymes generate high terpeneoid chemical diversity that has played a major role in plant diversification and adaptation. In this respect, an impressive new study led by Lichman, Buell, and O'Connor

has shed new light on the evolution of a prominent class of monoterpenes, namely iridoids, in a well-known aromatic plant family (the Lamiaceae) [5]. This investigation is a tour de force because it reveals the molecular mechanisms underlying the loss and re-emergence of iridoid biosynthesis in the *Nepeta* lineage.

The Evolution of Iridoids

Iridoids are produced by several plant families and act as a chemical defense against herbivores and plant pathogens. In the Lamiaceae (~200 genera, 7000 species), which comprise seven major subfamilies, iridoids are widely distributed across all subfamilies but are absent from a single subfamily, the Nepetoideae (Figure 1A). Importantly, because iridoids are also present in the sister family of the Lamiaceae, the Verbenaceae, it is likely that genes involved in iridoid biosynthesis evolved from a common ancestor, but the ability to produce iridoids has been lost in the clade of Nepetoideae. However, there is a noteworthy exception because iridoids are found in the Nepetoideae genus *Nepeta*. These plants are known as catmint or catnip owing to the euphoria-inducing effect of the nepetalactone iridoids on the behavior of felines. The intriguing presence of iridoids in Nepetoideae thus raises the question of whether the same enzymes or novel enzymes are involved in the re-emergence of iridoid biosynthesis (Figure 1A).

The structural core of iridoids is a *cis*- or *trans*-fused cyclopentanopyran. Unlike cyclic terpenoids that are generally produced by cyclization of a linear terpene carbocation by TS enzymes, the key step in iridoid biosynthesis is reductive cyclization of 8-oxogeranial by iridoid synthase (ISY) followed by either a Diels–Adler reaction or a Michael addition [6]. ISY was originally discovered in the Madagascar periwinkle (*Catharanthus roseus*, Apocynaceae), a major source of anticancer drugs derived from iridoid monoterpene indole alkaloids [6,7].