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Spotlight

Plant osmotic stress signaling: MAPKKKs meet SnRK2s

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Abstract (50 words).

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.

Main text (1258 words)

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 perceived by the cell as turgor loss and triggers signaling cascades for osmotic adjustment [Fig 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, 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, 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) showed partial constitutive activation of ABA-activated SnRK2s, whereas ABA treatment led to enhanced activation of the ABA-activated SnRK2s compared to wt. This suggested the presence of an unidentified positive mechanism for SnRK2 activation. Genetic studies led to the identification of the AR7 mutant, which was impaired in both ABA and OS response. 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.

RAF-like MAPKKKs 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 MAPKKKs. This family of kinases has greatly expanded in angiosperms and, for example, *Arabidopsis thaliana* contains 21 MEKK-like and 48 RAF-like MAPKKKs (10). In arabidopsis, group A comprises MAPKKKs 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 MAPKKKs by phylogenetic analyses based on their protein kinase catalytic domain, it is possible that biochemically 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 not necessarily reflects similar role in angiosperms; however, subsequent discoveries have established a functional link of B3-MAPKKKs, as well as B2- and B4-MAPKKKs, with SnRK2s in arabidopsis [2-6]. Given that B2/B3/B4 subgroups comprise 19 genes overall (Fig 1), high order mutants were required to genetically support their role in the activation of SnRK2s. A redundancy-circumventing genetic screen based on amiRNA-expressing lines that target gene families led to the isolation of MAPKKK amiRNA mutants able to germinate in the presence of 2 μM ABA [2]. Three B3-MAPKKKs, At5g11850/RAF3/M3Kδ1, At1g73660/RAF5/SIS8/M3Kδ6 and At1g18160/RAF4/M3Kδ7, reactivated previously dephosphorylated SnRK2.6 [2]. The *raf3/m3kδ1 raf4/m3kδ7 raf5/m3kδ6* triple mutant shows reduced sensitivity to ABA but surprisingly also reduced stomatal conductance, which is opposite to the phenotype of *snrk2.2/3/6* triple mutant and deserves further investigation [2]. Moreover, *raf3 raf4 raf5* is able to germinate on 2 μM ABA, but *snrk2.2/2.3/2.6* triple or *112458* pyl sextuple mutants are resistant even to 50 μM ABA. The amiRNA_m3k line targets 5 B3-MAPKKKs but still shows activation of SnRK2.2/2.3/2.6 in response to ABA [2].

Analysis of a decuple snrk2 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 OK^{100} as members of the B2/B3-MAPKKKs, whereas OK^{130} correspond to B4-MAPKKKs [3]. Inactivation of the seven B4-MAPKKKs does not affect OS-induced activation of subfamily III but abolish 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-MAPKKKs are UAKs for ABA-unresponsive subfamily I SnRK2.1/4/5/9/10. This finding is coincident with results from [4], who identified RAF18/20/24 as playing a major role for activation of subclass I SnRK2s. To identify those MAPKKKs that mediate OS-induced activation of SnRK2.2/3/6 the authors edited B2/B3-MAPKKKS to generate the *raf2/edr1 raf4 raf5 raf10 raf11* pentuple mutant, termed OK^{100}-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] (Fig 1).

A genetic 'tour de force' enabled the generation of a mutant impaired in 14 RAFs, including 4 B2-, 3 B3- and 7 B4-MAPKKKs (OK-quatdec). This mutant shows reduced sensitivity to 1 μM 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, *abi1-2/abi2-2/pp2ca-1* and *hab1-1/abi1-2/abi2-2* triple mutants 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 MAPKKKs actually function in the canonical MAPK module.

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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 (Ψw) 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 (Ψs) through accumulation of compatible solutes such as proline, osmotic adjustment can drive Ψw to values lower than soil Ψw. The pressure potential (Ψp, turgor is positive pressure and generates Ψp greater than 0 MPa) is compensated by lowering Ψs. 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 MAPKKKs. 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 MAPKKKs. ABA 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). The ABA glucosyl ester (ABA-GE), stored in vacuole or ER, is released in response to water deficit and represents another source of ABA. Subfamily I SnRK2s are activated by B4-MAPKKs, whereas subfamily III SnRK2s by B2- and B3- MAPKKKs. Full activation of 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. 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).

Glossary (198 words)

MAPKKK: Mitogen-activated protein kinase kinase kinase

MAPKK: Mitogen-activated protein kinase kinase

MAPK: Mitogen-activated protein kinase

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

Subfamily III SnRK2s: SnRK2.2/3/6, ABA-activated, OS-activated

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

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

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

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

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

***raf16 raf18 raf20 raf24 raf35 raf40/hcr1 raf42* septuple mutant**: impaired in 7 B4-MAPKKKs, also termed as OK^{130}-weak (residual activity of raf24 and raf42) or OK^{130}-null (null alleles)

***raf2/edr1 raf4 raf5 raf10 raf11* pentuple mutant**: impaired in 2 B2- and 3B3-MAPKKKs, also termed as OK^{100}-quin.

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

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

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

$abi1^{G180D}$, $abi2^{G168D}$ and $hab1^{G246D}$ dominant mutants: encode PP2Cs that are refractory to inhibition by ABA and ABA receptors

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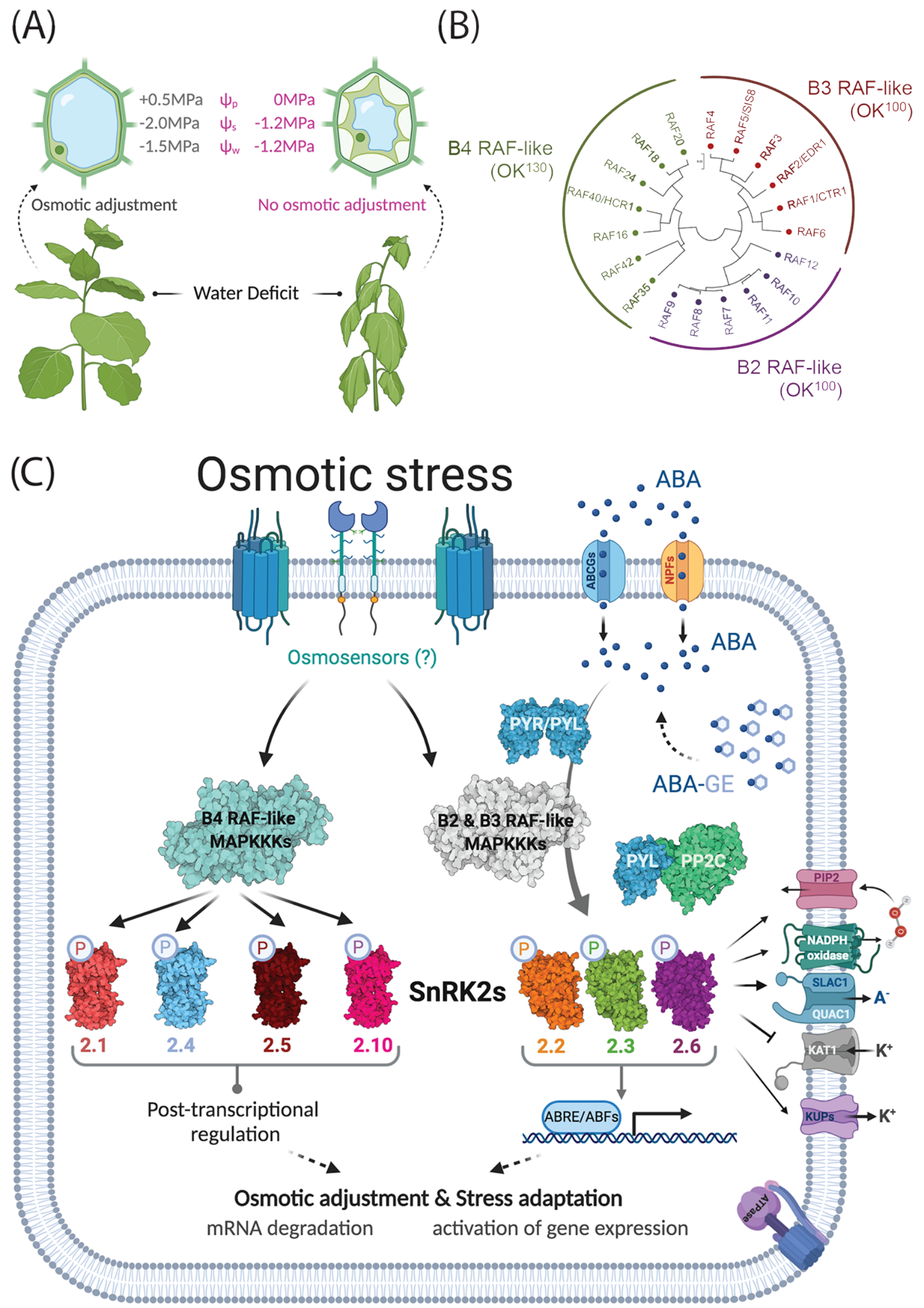
(A)
+0.5MPa ψp 0MPa
-2.0MPa ψs -1.2MPa
-1.5MPa ψw -1.2MPa
Osmotic adjustment
No osmotic adjustment
Water Deficit
(B)
B4 RAF-like (OK130)
B3 RAF-like (OK100)
B2 RAF-like (OK100)
RAF20
RAF4
RAF5/ISI8
RAF3
RAF2/EDR1
RAF1/CTR1
RAF6
RAF12
RAF10
RAF11
RAF7
RAF8
RAF9
RAF35
RAF42
RAF16
RAF40/HCR1
RAF24
RAF18
(C)
Osmotic stress
ABA
ABCGs
NPFs
ABA
Osmosensors (?)
PYR/PYL
ABA-GE
B4 RAF-like MAPKKKs
B2 & B3 RAF-like MAPKKKs
PYL
PP2C
PIP2
NADPH oxidase
SLAC1
QUAC1
A-
KAT1
K+
KUPs
K+
SnRK2s
2.1
2.4
2.5
2.10
2.2
2.3
2.6
Post-transcriptional regulation
ABRE/ABFs
Osmotic adjustment & Stress adaptation
mRNA degradation
activation of gene expression
ATPase