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REVIEW

EVOLUTION OF OSMOTIC STRESS SIGNALING *VIA* MAP KINASE CASCADES

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Summary

Cells respond to changes in osmotic pressure with compensatory molecular adaptations that allow them to re-establish homeostasis of osmotically disturbed aspects of cell structure and function. In addition, some cell types respond to osmotic stress by changing their phenotype or, if their tolerance threshold is exceeded, by initiating programmed cell death. To understand how cells achieve these different types of adaptive response to osmotic stress, it is necessary to identify the key elements of osmosensory signal transduction and to analyze the complex networks that process osmotic stimuli imposed upon cells by their environment. This review highlights mitogen-activated protein kinase (MAPK) cascades as important intracellular signal-transduction pathways activated in response to changes in osmolality. A unifying theme of osmotic stress signaling *via* MAPKs seems to be regulation of the cell cycle as part of the cellular stress response. This very important physiological capacity may have been conserved throughout evolution as a major function of MAPKs from many different subfamilies. The evidence for this conjecture is discussed, and our current knowledge about osmotic stress signaling pathways in yeast, animals and plants is briefly reviewed.

Key words: MAP kinase, osmotic stress, signal transduction, homeostasis, programmed cell death, molecular evolution.

Introduction

In many types of cells, osmotic stress interferes with cell volume and intracellular inorganic ion homeostasis. A notable exception are plant cells, which have rigid cell walls and can generate a turgor pressure. Most other cells shrink when exposed to hyperosmotic medium and swell in hypo-osmotic medium as a result of osmosis. The resulting change in cell volume is accompanied by an alteration in the intracellular density of macromolecules. Generally, cells cannot tolerate significant perturbations of cell volume or macromolecular density and they have the capacity to regulate their volume. Cell volume regulation is fast (generally within minutes) but leaves cells with disturbed intracellular inorganic ion concentrations that are in most cases disadvantageous. The concentrations of intracellular electrolytes (in particular Na^+ and K^+) are highly conserved across all kingdoms and phyla. This high level of conservation reflects an evolutionarily ancient optimization of the fundamental metabolic machinery of cells to function at a particular ionic milieu. Only a few species of halophilic and methanogenic archaebacteria are exceptional in this regard. Thus, cells restore their conserved ionic milieu, chiefly by adjusting the levels of compatible osmolytes (Somero and Yancey, 1997).

Because cell volume and ion regulation are not instantaneous processes, osmotic stress may damage cellular macromolecules and impair cell function until compensatory adaptations counteract the stress. Damage to DNA and proteins leads to impairment of cell function and to the induction of repair processes and protection systems (Naegeli, 1997). This relatively nonspecific ‘cell damage response’ may be an important aspect of the cellular adaptation process following osmotic stress and, in concert with cell volume regulation and ionoregulation, may serve to compensate for negative osmotic effects on cell integrity and function. It is now becoming increasingly recognized that, if cells are no longer able to compensate for osmotic stress and the amount of damage is too great, they trigger a self-destruction program called programmed cell death (apoptosis) (Schwartz and Osborne, 1993; Katsuhara, 1997). The selective advantage of such a mechanism lies in the elimination of malfunctioning and potentially malignant cells from the organism.

Finally, certain cell types of osmoregulatory epithelia respond to changes in environmental osmolality with phenotypic changes that alter the direction and/or capacity of transepithelial ion transport. Examples are the mitochondria-rich cells of mammalian renal or teleost gill epithelia (Schwartz *et al.* 1985; Jürss and Bastrop, 1995). Regulation of cell

phenotype by osmolality is part of an integrative response at the level of the whole organism aimed at preserving the osmotic homeostasis of body fluids in vertebrates and other osmoregulating animals. Osmotically induced changes in cell phenotype are often based not only on primary osmotic signals but also on additional secondary stimuli such as neural, hormonal and paracrine signals that serve as messengers of signal–response integration at the level of the whole organism (Dunel-Erb *et al.* 1982; Foskett *et al.* 1983).

This review summarizes our current knowledge about the evolution of mitogen-activated protein kinases (MAPKs) as transducers of osmotic signals and discusses what we have learned about their role in the different aspects of the cellular osmotic stress response briefly outlined above.

Molecular evolution of MAP kinases

The MAP kinase family is a large family of proteins and belongs to the CMGC group of the eukaryotic protein kinase superfamily (Hanks and Hunter, 1995). The CMGC group contains four protein families with a high degree of similarity: Cyclin-dependent kinases, MAP kinases, Glycogen synthase kinases 3 and Cdc-like kinases. All MAPKs are characterized by the unique signature motif [LIVM][TS]*XX*[LIVM]*X*T[KR][WY]YR*X*P*X*[LIVM][LIVM] that distinguishes them from other eukaryotic protein kinases (Kültz, 1998). This signature motif consists of part of the phosphorylation lip and the 'P+1' substrate-binding pocket, both of which are located on loop 12 of the three-dimensional structure of the MAP kinase and are critical for MAPK activity. MAPKs are crucial for many aspects of cell physiology, including regulation of the cell cycle, cell growth and differentiation, cell death and cellular pathogen defense mechanisms. They are central elements of conserved phosphorylation cascades that represent major signal-transduction pathways extending from the cell surface into the nucleus (Treisman, 1996).

A radial phylogenetic tree reconstructed by using MAPK sequences from yeast, slime molds, protozoa, plants, invertebrates and vertebrates is depicted in Fig. 1. This tree illustrates the three major subgroups of MAPKs: extracellular-signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs) and the MAPK3 subgroup. On the basis of their phylogenetic relationships and functional characteristics, it is possible to distinguish at least nine MAPK subfamilies, five of which are ERKs, three are SAPKs and one is a MAPK3 (Kültz, 1998). Several protozoan MAPKs and some unique sequences from *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Dictyostelium discoideum* are likely to be founding members of additional MAPK subfamilies (Fig. 1). Members of a given subfamily are exclusively from plants, animals or fungi. Each subfamily except MAPK3 is characterized by a conserved dual phosphorylation motif T*X*Y (threonine – variable amino acid – tyrosine) representing the activation site.

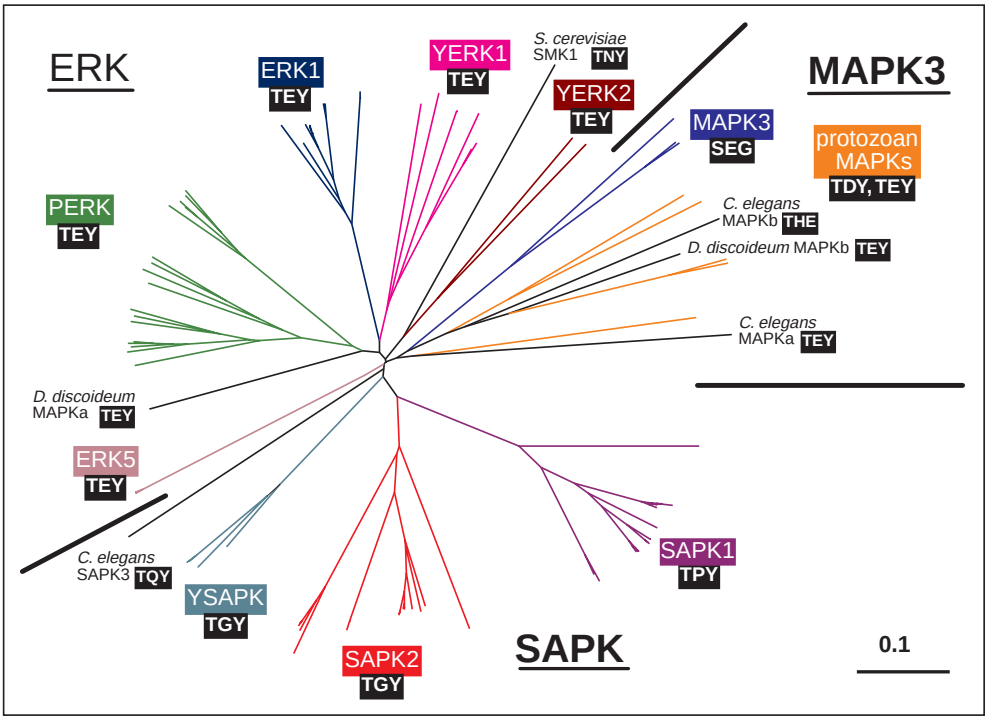

Fig. 1. Radial phylogenetic tree of the mitogen-activated protein kinase (MAPK) family. The MAPK family contains three subgroups: extracellular-signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs) and the MAPK3 subgroup. Subfamily names and their tree branches are color-coded, and the dual phosphorylation motif is illustrated by inverse printing immediately underneath the name of each subfamily and potential founding members of additional subfamilies. The ERK subgroup contains at least five subfamilies: animal ERK1 (including MAPK1=ERK1=p44 and MAPK2=ERK2=p42) and ERK5 (MAPK5, BMK1), the yeast MAPK subfamilies YERK1 and YERK2, and the plant MAPK subfamily PERK. All ERKs, except *Saccharomyces cerevisiae* SMK1, have a TEY dual phosphorylation motif in the activation loop. The SAPK subgroup consists of the animal subfamilies SAPK1 (JNK) and SAPK2 (p38, RK, CSBP, Mxi2, MPK2) and the yeast subfamily YSAPK (HOG). The MAPK3 subgroup contains the animal MAPK3 subfamily and many protozoan MAPKs. Note that the MAPK3 subfamily and *Caenorhabditis elegans* MAPKb have no dual phosphorylation motif and are exceptional MAPKs in this regard. A bar for calibration of phylogenetic distances (K_{aa}) is provided [$K_{aa}=-\ln(1-D-D^2/5)$, where D is the observed distance between 2 sequences].

But not every subfamily contains a unique variable amino acid (*X*) within the T*X*Y motif, and a few MAPKs have only single phosphorylation motifs. Almost all members of the ERK subgroup and many members of the MAPK3 subgroup have TEY (Thr-Glu-Tyr) dual phosphorylation motifs. Therefore, it is likely that the MAPK3 and ERK subgroups emerged from a common ancestral MAPK bearing a TEY motif before protozoa and slime molds diverged from the phylogenetic lineages that gave rise to plants, animals and fungi. On the basis of these considerations, it is likely that the MAPK family is at least 1–1.5 billion years old and appeared relatively soon after the origin of the eukaryotes. SAPKs have only been found in animals and yeast. This may indicate that the SAPK subgroup is phylogenetically the youngest MAPK group. It may have split from the ERK lineage after the protozoan and plant lineages had separated from the metazoan/fungal lineage.

Having considered very briefly the basic phylogeny and molecular evolution of MAPKs, we now discuss the osmotic responsiveness of MAPKs from individual subfamilies and attempt to reconstruct how osmotic stress signaling *via* MAPKs has evolved.

Yeast SAPKs: the high-osmolarity glycerol response pathway

The high-osmolarity glycerol (HOG) response pathway was first discovered in the budding yeast *S. cerevisiae* (Brewster *et al.* 1993). Many homologous elements of this pathway in *Schizosaccharomyces pombe* and *Candida albicans* were subsequently cloned. The major components of the HOG pathway in *S. cerevisiae* are depicted in Fig. 2. The MAPK in this pathway, HOG1, occupies the central position within a kinase cascade of three highly conserved elements: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. Interestingly, the HOG cascade is positioned immediately downstream of a 'two-component system' consisting of a primary osmosensor (SLN1), an intermittent phosphorelay protein (YPD1) and a response regulator protein (SSK1) (Wurgler-Murphy and Saito, 1997). Hyperosmotic induction of the HOG pathway has been reviewed previously (Wurgler-Murphy and Saito, 1997) and can be briefly summarized as follows. At normal osmolality, the osmosensor SLN1 is constitutively active and phosphorylates the response regulator SSK1. Phosphorylation activates SSK1 which, in turn, represses the activity of two redundant MAPKKKs: SSK2 and SSK22. This repression turns the MAPK cascade off and results in HOG1 being inactive. Hyperosmolality apparently inhibits SLN1 kinase activity, thereby activating the HOG cascade.

Two-component systems are very common elements of signal transduction in prokaryotes and they have only recently been discovered in fungi and plants. In *E. coli* and other bacteria, the primary osmosensor EnvZ and the response regulator OmpR, which also acts as an inducible transcription factor, are the only two proteins required for transducing information about osmotic stress from the inner cell membrane

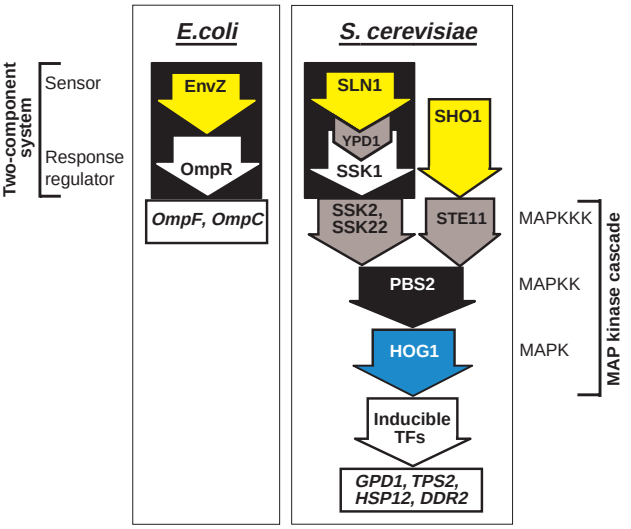

Fig. 2. Schematic representation of hyperosmotic stress signaling pathways in bacteria and yeast. Primary osmosensors are depicted in yellow and the MAP kinase in blue. A two-component system consisting of the EnvZ osmosensor and the OmpR response regulator transmits information about osmotic stress from the cell surface to the level of gene expression in the bacterium *Escherichia coli*. A homologous two-component system, consisting of the SLN1 osmosensor and the SSK1 response regulator, monitors osmotic stress in the yeast *Saccharomyces cerevisiae*. However, in contrast to bacteria, a MAP kinase cascade is required as an additional signaling module to carry the information about osmotic stress to the level of gene expression. This MAP kinase cascade can also be osmotically regulated *via* the alternative osmosensor SHO1. For a discussion of the function of the target genes of this MAP kinase pathway, refer to the text.

to *cis* elements of specific target genes, hence the name 'two-component system' (Fig. 2). The *OmpF* and *OmpC* genes are regulated by this two-component system. They encode porins that are important for the regulation of cell membrane permeability. Even though two-component systems have not been found in animals, the extension of such a signaling unit by the HOG cascade in yeast is an indication of the increasing degree of sophistication of osmotic stress signaling in eukaryotes. An alternative osmosensor, SHO1, can also regulate the yeast HOG cascade independently of the SLN1/SSK1 two-component system (Fig. 2). SHO1 regulates HOG1 *via* the MAPKKK STE11 (Posas and Saito, 1997). Thus, it could be envisioned that, in higher eukaryotes, two-component systems might have been bypassed and replaced by alternative osmosensors. Conversely, the yeast SLN1/SSK1 two-component system is also linked to pathways other than the HOG cascade, including one that controls the activity of the Mcm1 transcription factor (Yu *et al.* 1995). Among the targets of the HOG pathway are important inducible transcription factors such as ATF1 and Msn2/4. These transcription factors induce the expression of osmolyte-synthesizing genes, e.g. *GPD1* (encoding glycerophosphate dehydrogenase 1) and *TPS2* (encoding trehalose phosphate phosphatase) (Ruis and Schüller, 1995). The resulting increase

in the levels of the compatible osmolytes glycerol and trehalose leads to the replacement of excessive inorganic ions and the restoration of intracellular electrolyte homeostasis in situations of hyperosmotic stress. Other target genes of the HOG pathway include *HSP12* and *DDR2* (Ruis and Schüller, 1995). *HSP12* encodes a molecular chaperone that presumably assists in compensating osmotic damage to cellular proteins. *DDR2* (encoding DNA damage-responsive gene 2) may be involved in DNA damage recognition/repair. Finally, the HOG pathway is important for the coordinated control of the progression of the cell cycle with the cellular response to osmotic stress (Shieh *et al.* 1998). The next paragraph will outline the emerging view that integration of cell cycle regulation with the cellular osmotic stress response represents a major element of osmotic stress signaling *via* animal SAPKs.

Osmotic regulation of animal SAPKs

Only a single SAPK-encoding gene (the gene for HOG1) is contained in the fully sequenced genome of the yeast *S. cerevisiae*. In contrast, animal genomes encode members of multiple SAPK subfamilies. The genome of the primitive invertebrate *C. elegans* contains one gene for SAPK1, one gene for SAPK2 and another gene for SAPK3 that is presumably a founding member of a new SAPK subfamily (Fig. 1). A truly amazing diversification of SAPK isoenzymes is apparent in vertebrate genomes. Humans, for instance, have at least three genes for SAPK1 and four for SAPK2, most of which have splice variants. SAPKs are strongly activated by cytokines and are important for T-cell-based immunity and inflammation; their broad diversification is paralleled by the development of a system of acquired immune response in vertebrates.

Besides their importance for cytokine signaling, the SAPK1 and SAPK2 pathways are strongly activated by hyperosmotic stress. Some SAPK1 and SAPK2 isoenzymes are able functionally to complement *S. cerevisiae* ΔHOG1 mutants and rescue these yeast cells from death in hyperosmotic medium (Galcheva-Gargova *et al.* 1994; Han *et al.* 1994). Targets of the SAPK1 pathway include important inducible transcription factors such as c-Jun and Elk1. Likewise, the SAPK2 pathway also controls the activity of important inducible transcription factors, including ATF2 and MEF2c. However, not all the genes regulated by these transcription factors are induced by osmotic stress. Thus, it appears that in osmotically stressed animal cells there is cross talk between SAPKs and other pathways to integrate osmotic stress signals and yield stressor-specific adaptations. This phylogenetic trend towards signal integration and networking is apparent from a comparison of osmotic stress signaling in prokaryotes and yeast (Fig. 2).

A multitude of MAPK pathways, including SAPK1 and SAPK2, are hyperosmotically induced in vertebrate cells. However, the SAPK1 and SAPK2 pathways are also responsive to a wide variety of other cellular stresses, e.g. ultraviolet and γ irradiation (SAPK1), or to oxidative stress and to the inhibition of protein synthesis (SAPK2). At present, no animal homologues of bacterial and yeast two-component systems have been found and no other primary osmosensors that regulate SAPK pathways have been identified. Potential candidates for primary osmosensors include intracellular solute sensors, membrane-based osmosensors and cytoskeleton-associated osmosensors (Kültz and Burg, 1998). Currently, there is contradictory evidence concerning the requirement of MAPK pathways for the organic osmolyte response in animal cells (Kwon *et al.* 1995; Kültz *et al.* 1997; Wojtaszek *et al.* 1998; Sheikh-Hamad *et al.* 1998). This problem needs to be addressed further in more detail using a broad array of methodological approaches. However, it is well established that SAPKs are very important for cell cycle regulation, for the control of cellular integrity/repair and for the induction of programmed cell death. The duration of SAPK1 activation in human Jurkat T cells and 293T embryonic kidney cells determines the fate of these cells in response to environmental stress (Chen *et al.* 1996). Moreover, the SAPK2 pathway contributes to hyperosmotic induction of GADD45 and GADD153 expression in murine kidney cells (Kültz *et al.* 1998). *GADD45* and *GADD153* are growth-arrest- and DNA-damage-inducible genes that participate in the recognition and/or repair of DNA damage. These observations are in agreement with hyperosmotic induction of growth arrest of murine kidney cells in G2 (Kültz *et al.* 1998). This stagnation of cell growth may be critical for minimizing the impact of stress and/or to assess the severity of the stress, i.e. the extent of damage to cellular proteins and DNA. Cells may continue to proliferate or differentiate only if macromolecular damage caused by environmental stress can be matched by their repair capacity. If the amount of damage to proteins and/or DNA is too high, cells can activate a program of cell death to eliminate themselves from the organism. The adaptive benefit for the whole organism lies in the removal of malfunctioning cells with defective macromolecules, which could potentially become malignant and tumorigenic. Hypermutability and stable changes in karyotype and phenotype as a result of osmotic stress have indeed been observed in cultured mammalian kidney cells (Uchida *et al.* 1987).

The YERK2 subfamily: hypo-osmotic activation of the cell wall integrity pathway

Many ERK isoforms from diverse subfamilies, including YERK2, ERK1, ERK5 and PERK, are activated in response to osmotic stress. In yeast, hypo-osmolality triggers a cell wall integrity pathway, the central element of which is the MAP kinase MPK1 (Ruis and Schüller, 1995). MPK1 is the name of this enzyme in *Schizosaccharomyces pombe*, whereas homologous enzymes have been termed SLT2 in *S. cerevisiae* and MKC1 in *Candida albicans*. All three of the aforementioned MAPKs have very similar functions and activation profiles, and they all belong to the YERK2 subfamily (Kültz, 1998). The primary activation signal for MPK1 seems to be a defect or change in cell wall integrity that can have multiple causes, among them cell swelling in response to hypotonicity, heat shock, bud morphogenesis,

polar cell growth or other abnormalities in cell morphology. The MPK1 pathway targets proteins involved in the regulation of gene expression during the G1/S transition in the yeast cell cycle (Madden *et al.* 1997). Moreover, hypo-osmotic activation of the MPK1 pathway causes growth arrest in G2 (Mizunuma *et al.* 1998). This stress-induced delay of mitosis resembles the hyperosmotically induced G2 growth arrest of murine kidney cells (see above) and seems to represent a phylogenetically conserved response of cells to osmotic stress. Growth arrest may have a selective advantage to cells insofar as (1) energetic resources could be channeled into specific adaptive mechanisms, e.g. the organic osmolyte response, adaptive differentiation, etc., and (2) cell metabolism may slow down and DNA/protein damage may be kept to a minimum while repair mechanisms are activated to avoid proliferation of cells with a damaged genotype and/or phenotype. Growth arrest may also be necessary for cells to assess the severity of osmotic stress and, on the basis of the amount of macromolecular damage detected, initiate either appropriate repair mechanisms or programmed cell death. MAPK pathways are crucial for signaling the maintenance of cellular integrity or, alternatively, programmed cell death in response to environmental (including osmotic) stress. Therefore, MAP kinases may be at the heart of this cellular decision-making process.

Osmotic regulation of animal ERKs

Animal extracellular-signal-regulated kinases (ERKs) comprise two distinct subfamilies, ERK1 and ERK5 (Fig. 1). Both subfamilies are regulated by osmotic stress. Activation profiles and the physiological significance of ERK1 have been characterized much more extensively than for ERK5. Nevertheless, it has been shown that ERK5 is activated by hyperosmolality in rat vascular smooth muscle cells (Abe *et al.* 1996).

The ERK1 subfamily consists of two paralogous isoforms, ERK1α and ERK1β. Both ERK1 isoforms are responsive to a wide variety of stimuli, but primarily to mitogens, such as growth factors. Nevertheless, hyperosmotic stress also activates ERK1 in many mammalian cell types. In addition, hypo-osmolality has been shown to activate ERK1 in H4IIE hepatoma cells (Schliess *et al.* 1996). In MDCK cells, hyperosmotic activation of ERK1 is dependent on protein kinase C, but inhibition of ERK1 activation by the protein kinase C inhibitor 12-*o*-tetradecanoylphorbol 13-acetate does not affect hyperosmotic induction of *myo*-inositol or glycine betaine transporter genes (Kwon *et al.* 1995). Studies on murine kidney cells have shown that hyperosmotic stress in the form of high urea levels induces an ERK1-dependent activation of the immediate early genes *c-fos* and *egr-1* (Cohen, 1996). The activation of the *egr-1* gene is specific for the hyperosmotic stress caused by high urea levels and not that caused by high levels of NaCl. Although the physiological role of ERK1 activation remains largely unclear, the ERK1 pathway may be important for the adaptive differentiation of inner medullary kidney cells and for adjusting renal transport to situations of osmotic stress.

Hyperosmotic activation of the ERK1 pathway is specifically suppressed by mitogen-activated protein kinase phosphatase 1 (MKP1), which is induced *via* the SAPK2 pathway (Schliess *et al.* 1998). This negative feedback mechanism between two osmotically activated MAPK pathways appears to be beneficial considering the fact that ERK1 activation mostly promotes cell proliferation, whereas SAPKs often signal cell cycle delay or programmed cell death. At present, the question of why osmotic stress activates more than one MAPK in a single cell remains unanswered. One reason could be that an integration of cross talk between multiple osmotic stress signaling pathways might contribute to confer stressor-specificity to the adaptive strategy of cells.

PERKs: signaling drought and water stress

In plants, the MAPK family is represented by only a single subfamily (Fig. 1). This subfamily belongs to the ERK subgroup, hence the subfamily name plant ERKs (PERKs). PERKs are involved in signaling cell cycle regulation, plant ovule development and wound healing. Among the diverse activation signals for PERKs are auxin, cold stress and gibberellin. Since no SAPKs have been found in plants, PERKs may have evolved the capacity to carry stress signals that are transmitted by SAPKs in yeast and animals. Several PERKs are strongly activated by osmotic stress. In *Medicago sativa*, drought activates MMK4, a PERK with a relative molecular mass of 44 (Jonak *et al.* 1996). Interestingly, MMK2, which is a paralogous homologue of MMK4, is able specifically to complement yeast ΔMPK1 mutants (see discussion of the YERK2 subfamily above), suggesting functional similarities between MMK2 and MPK1 osmotic stress signaling pathways (Jonak *et al.* 1995). Additional PERKs activated by osmotic stress include *Arabidopsis thaliana* ATMPK3 (Mizoguchi *et al.* 1996) and *Pisum sativum* PsMAPK (Popping *et al.* 1996). The latter is able to rescue yeast ΔHOG1 mutants in hyperosmotic medium (Popping *et al.* 1996), suggesting a close functional relationship between this PERK and a yeast SAPK. This observation supports the idea that certain PERKs are involved in aspects of osmotic stress signaling that are specific to the SAPK subgroup in animals and yeast.

Conclusions

Osmotic stress signaling *via* MAP kinase cascades is a phylogenetically ancient signaling mechanism in eukaryotes that has been retained in extant MAPK pathways found in yeast, animals and plants. This mechanism may have evolved by the extension of existing signaling modules in prokaryotes (two-component systems) after the origin of the eukaryotic protein kinase superfamily. With the evolution of metazoans, multicellular plants and vertebrates, MAPK cascades have been adopted as signal-transduction mechanisms necessary for intercellular communication and immunity, in addition to

carrying information about developmental processes and environmental stress. Nevertheless, almost all the MAPK subfamilies have retained a critical importance for signaling osmotic stress. In this capacity, they not only represent linear pathways but are also part of a highly integrated signal-transduction network that coordinates cellular (and organismal) responses to environmental stimuli. The outcome of this cellular information processing system seems to be a coordination of the cell cycle and cellular differentiation in response to environmental stress, ultimately resulting in an optimization of organismal fitness. Although much information about the role of osmotic stress signaling by MAPKs has been gathered, many questions remain unanswered. In the future, we need to identify the primary osmosensors that regulate MAPKs in animals and plants, we must gain insight into the main molecular circuits by which cell cycle regulation is achieved in response to osmotic stress and we need to learn how specificity arises from osmotic stress signaling *via* MAPKs to result in adaptive stress compensation and phenotypic changes of cells.

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