Abiotic stress signalling pathways: specificity and cross-talk

Heather Knight and Marc R. Knight

Plants exhibit a variety of responses to abiotic stresses that enable them to tolerate and survive adverse conditions. As we learn more about the signalling pathways leading to these responses, it is becoming clear that they constitute a network that is interconnected at many levels. In this article, we discuss the 'cross-talk' between different signalling pathways and question whether there are any truly specific abiotic stress signalling responses.

Plants encounter a wide range of environmental insults during a typical life cycle and have evolved mechanisms by which to increase their tolerance of these through both physical adaptations and interactive molecular and cellular changes that begin after the onset of stress. The first step in switching on such molecular responses is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway. These pathways eventually lead to physiological changes, such as guard cell closure, or to the expression of genes and resultant modification of molecular and cellular processes. Our knowledge about the signalling pathways leading from stimulus to end response in plants has increased over recent years. It is increasingly apparent that the linear pathways that we have been studying are actually only part of a more complex signalling network and that there is much overlap between its branches, with, for instance, many genes inducible by more than one particular stimulus.

In this article, we discuss two aspects of these abiotic stress signalling networks, namely cross-talk and specificity. We define 'cross-talk' as any instance of two signalling pathways from different stressors that converge. This might take the form of different pathways achieving the same end or of pathways interacting and affecting each other's outcome, including the flux through one pathway affecting another. These might act in an additive or negatively regulatory way, or might compete for a target (Fig. 1). We define specificity as any part of a signalling pathway that enables distinction between two or more possible outcomes and that thus might link a particular stimulus to a particular end response and not to any other end responses. Opportunities for both cross-talk and specificity can occur within a particular pathway.

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Cross-talk

When stress signalling pathways are examined in the laboratory, they are usually considered in isolation from other stresses to simplify interpretation. In nature, however, the plant encounters stress combinations concurrently or separated temporally and must present an integrated response to them. In the case of phytochrome signalling, the two pathways leading to red-light-induced *CHS* and *CAB* gene expression negatively regulate flux through one another[1,2]. Seemingly separate abiotic stress signalling pathways are also likely to interact in a similar manner. In addition, several abiotic stress pathways share common elements that are potential 'nodes' for cross-talk. Cross-talk can also occur between pathways in different organs of the plant when a systemic signal such as hydrogen peroxide moves from a stimulated cell into another tissue to elicit a response[3].

Specificity

In spite of considerable overlap between many abiotic stress signalling pathways, there might, in some instances, be a benefit to producing specific, inducible and appropriate responses that result in a specific change suited to the particular stress conditions encountered. One advantage would be to avoid the high energy cost of producing stress-tolerance proteins, exemplified by the dwarf phenotype of plants constitutively overexpressing the frost tolerance protein DREB1A (Ref. 4). In some cases, the signal transduction pathways triggered by different stresses are common to more than one stress type. One possible reason for this is that, under certain conditions, the two stresses cannot be distinguished from one another. Alternatively, each stress might require the same protective action (or at least some common elements). The discovery of separate sensing mechanisms for each stress would invalidate the first suggestion but the second is true in several cases. For example, dehydration protection is required in plants undergoing either freezing or drought and the production of antioxidants and scavenging enzymes (e.g. catalase and peroxidases) that protect against oxidative damage affords protection against a variety of different abiotic (and biological) stresses[5].

Most abiotic stresses tested have been shown to elicit rises in cytosolic free calcium levels ($[Ca^{2+}]_{cyt}$) and to involve protein phosphatases and kinases [including mitogen-activated protein kinase (MAPK) cascades]. However, are any of these components truly specific to one stress and which of them are 'nodes' at which cross-talk occurs? In the following sections, we consider different classes of signalling component in turn, and examine their potential

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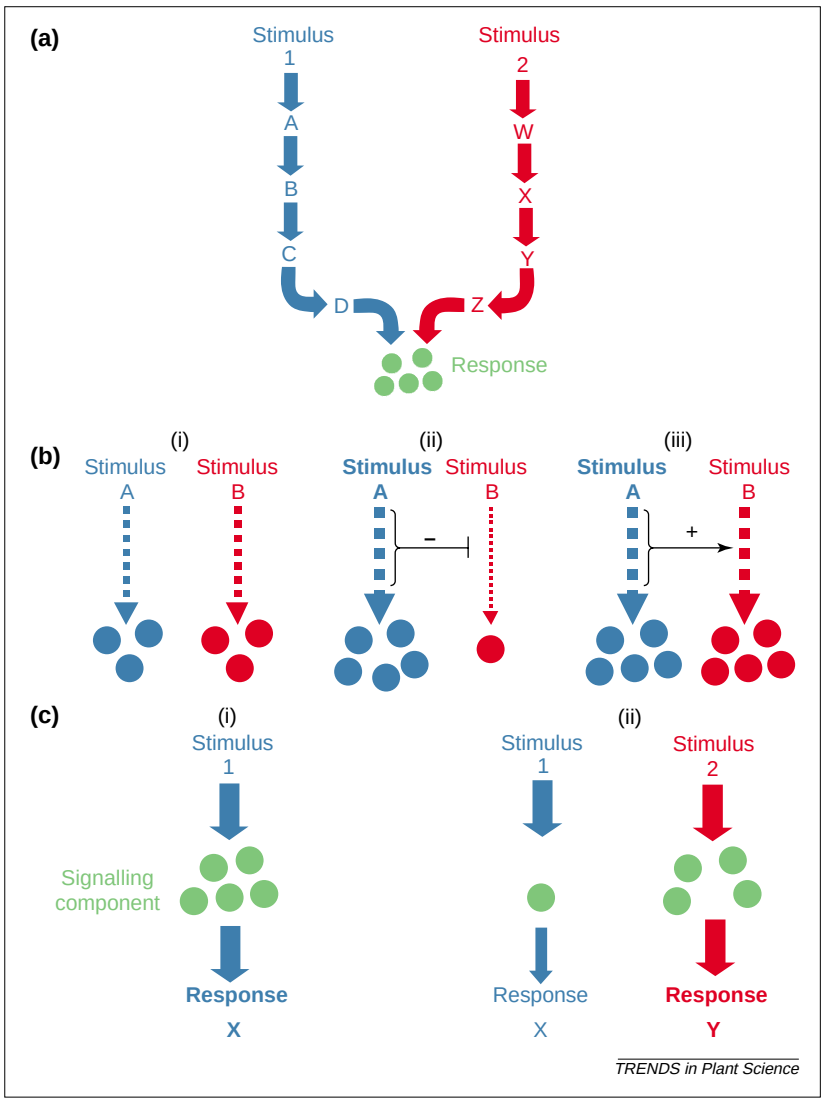

Fig. 1. Cross-talk in signalling pathways. (a) Two different stimuli (1 and 2) evoke the same end response via different signalling pathways, using different signalling intermediates (A–D and W–Z, respectively). (b) Positive and negative reciprocal control. Two different stimuli (A and B) activate two signalling pathways (broken arrows), leading to different end responses. (i) Pathways operating totally independently of each other. (ii) Flux through the stimulus-A-mediated pathway negatively regulates the stimulus-B-mediated pathway and inhibits its flux. An example of this is in phytochrome-mediated expression of a chlorophyll *a*/*b* binding protein gene (*CAB*) and a chalcone synthase gene (*CHS*) by independent pathways, each negatively regulating the other. (iii) Flux through the stimulus-A-mediated pathway positively regulates the stimulus-B-mediated pathway and promotes its flux. (c) (i) Signalling pathway leading from a stimulus (stimulus 1) using a specific signalling component (green) to effect response X. (ii) Stimulus 2 uses this same signalling component to mediate its end response (response Y) and, by out-competing the stimulus-1 pathway, inhibits it. Calcium is an example of this: one stimulus might exhaust a specific pool of calcium and make it unavailable for use by another stimulus.

contribution to specificity and cross-talk between abiotic stress signalling pathways.

Sensing systems

Specificity might occur at the point of initial stress perception itself. In the case of osmotic stress, the putative osmosensor AtHK1 (Ref. 6), a transmembrane histidine kinase, is thought to be the first component to relay changes in osmotic potential outside the cell to the transduction pathway(s) inside the cell that regulates drought-inducible gene expression. If specific stresses are actually sensed by dedicated receptor molecules, these molecules themselves have the potential to encode specificity of response. An early event in the response to many different environmental stresses is an elevation in $[Ca^{2+}]_{cyt}$ (Refs 7,8), which is thought to be the primary stimulus-sensing event for several stresses (e.g. cold)[9–11]. If this is the case then mechanisms could exist for encoding the information that relates to the particular stress through the calcium signature (see below). Alternatively, the stress might be sensed through other components either in parallel to or upstream of Ca^{2+} in the pathway. It has been postulated that cold is sensed via changes in membrane fluidity[12] and cytoskeletal reorganization[13] affecting calcium channels.

Calcium

The precise kinetics, magnitude and cellular source of stimulus-induced $[Ca^{2+}]_{cyt}$ elevations (the 'calcium signature') have been proposed to encode information about the particular stimulus, and to determine the specific end response elicited[14]. Biphasic elevations, responses lasting from two seconds to tens of minutes and repeated oscillations are among the responses observed after abiotic stress.

Studies using animal cells showed that the Ca^{2+}-induced activation of particular transcription factors could be specified by the magnitude and kinetics of an artificially induced $[Ca^{2+}]_{cyt}$ elevation[15]. This suggests that cells can decode specific information in the $[Ca^{2+}]_{cyt}$ elevation that refers to particular stimuli, and relate this to an appropriate change in response. However, such data do not prove that this system of encoding specificity is actually used by cells. Also, these measurements were all made on populations of cells, obscuring the complexity of individual cell responses.

In plants, the principle of specificity through calcium signatures has been difficult to show because of problems in generating artificial $[Ca^{2+}]_{cyt}$ elevations to meet specific designs. In some cases, $[Ca^{2+}]_{cyt}$ has been successfully elevated in the absence of an abiotic stress by using a Ca^{2+} ionophore or Ca^{2+} channel agonists[16,17]. The correlation of such $[Ca^{2+}]_{cyt}$ elevations with changes in the end responses usually associated with exposure to the stress shows a requirement for Ca^{2+} in the transduction of these stimuli. However, it also supports the argument that specificity is not encoded through the calcium signature because it is most unlikely that the artificial $[Ca^{2+}]_{cyt}$ elevations could have the same subcellular source and calcium signature as those normally provoked by that particular stress. It is more likely that the $[Ca^{2+}]_{cyt}$ elevation must achieve a minimum (and perhaps maximum) threshold peak value or total elevation (magnitude × time). Ozone exposure elicits a brief $[Ca^{2+}]_{cyt}$ peak followed by a more prolonged elevation[18]. Only the second of these is necessary for the induction of *GST* gene expression and so, even though these data might imply the significance of the calcium signature, they might also imply that the 'magnitude × time' hypothesis is true.

In stomatal guard cells, variation in the timing of stimulus-induced Ca^{2+} oscillations has been correlated with the intensity of both the stimulus and the resultant end response, with alterations in the signature associated with loss of aperture closure[14,19]. External Ca^{2+} or oxidative stress elicited Ca^{2+} oscillations followed by stomatal closure in the wild type, but cells of the *det3 Arabidopsis* mutant (which has impaired endomembrane energization) failed to show normal wild-type oscillations and did not close. However, *det3* cells responded normally to cold and abscisic acid (ABA) stimulation, indicating that there are specific Ca^{2+}-dependent pathways for different stresses. Concurrent addition of external Ca^{2+} and repeated depolarization of the plasma membrane induced artificial Ca^{2+} oscillations in *det3* cells and initiated stomatal closure. These data suggest that Ca^{2+} oscillations are required for stomatal closure to occur in response to certain signals. Interestingly, the non-oscillatory stimulus-induced $[Ca^{2+}]_{cyt}$ elevations seen in *det3* mutants constituted a larger overall total increase in $[Ca^{2+}]_{cyt}$ than did the wild-type oscillations. This might support the idea that the response is not achieved if the total $[Ca^{2+}]_{cyt}$ elevation exceeds a certain level[15]. It should also be borne in mind that the *det3* $[Ca^{2+}]_{cyt}$ elevation might occur in the wrong cellular location. Various plant abiotic stress $[Ca^{2+}]_{cyt}$ responses use Ca^{2+} from different subcellular sources, including the extracellular compartment, vacuole[20] and mitochondria[21]. The Ca^{2+} signature reflects the source used[22] and might encode information of specific relevance to the cellular machinery based in those organelles.

It is possible that 'effective' Ca^{2+} signatures only occur in those cell types that are required to respond. $[Ca^{2+}]_{cyt}$ elevations occur globally in plants responding to cold but only in the root after drought[22] (S. Scrase-Field and M.R. Knight, unpublished). Within the *Arabidopsis* root, the $[Ca^{2+}]_{cyt}$ responses of epidermal, endodermal, pericycle and cortex cells differ from each other when challenged with cold, drought and salt[23]. These tissue-specific differences might correlate with different stress protein production or other responses.

Although plants exhibit recognizable $[Ca^{2+}]_{cyt}$ elevations in response to particular stresses, these are altered markedly after previous stress experiences[8,24], indicating cross-talk between abiotic stress signal transduction pathways occurring at the level of Ca^{2+}. Whereas an oxidative stress encounter abolished future responses to drought stimulus in *Arabidopsis*[24], it increased the sensitivity of response to low temperature levels[8]. Drought pretreatment increased the magnitude of subsequent drought-induced $[Ca^{2+}]_{cyt}$ transients and increased the level of drought-inducible Ca^{2+}-regulated gene expression and stress tolerance[24]. In summary, the Ca^{2+} signal is ubiquitous in abiotic stress signalling and it is therefore an important node at which cross-talk can occur.

Calcium-regulated proteins

$[Ca^{2+}]_{cyt}$ elevations achieve control of various processes via Ca^{2+}-regulated effector proteins. Sometimes referred to as 'calcium sensors', these include calmodulin, calcium-dependent protein kinases (CDPKs) and calcium-regulated phosphatases. Calmodulin has been implicated in plant responses to cold[25], mechanical stimulation[25,26] and oxidative stress[27]. The use of different isoforms could be involved in control of specificity between these pathways, as has been observed with calmodulin isoforms in specificity to biotic stresses such as salicylic-acid-mediated defence responses[28]. In animal cells, anchoring proteins located in different parts of the cell compartmentalize kinases to their site of action[29]. It is possible that specificity of response in plant systems, too, is introduced by localizing calcium sensors in this way.

Only two out of eight *Arabidopsis* CDPK isoforms introduced into maize protoplasts induced expression of a specific stress-inducible gene, suggesting that there are specific CDPK isoforms for different stress signalling pathways[17]. However, these experiments involved the overexpression of constitutive versions of CDPK isoforms, which, lacking all but the kinase domains of the proteins, might not target appropriately. Also, because they are ectopically expressed, they might phosphorylate illegitimate substrates. Therefore, these results might not reflect the *in vivo* situation in *Arabidopsis*. There are many different CDPKs in *Arabidopsis* [~40 (Ref. 30)], therefore there is ample scope for partitioning of specific CDPK function between isoforms. The genes for some isoforms are induced in response to specific stresses, implicating these CDPKs in particular signalling pathways; thus, for example, AtCDPK1 and AtCDPK2 are implicated in salt and drought stress signalling[17,31]. In rice, expression of *OsCDPK7* at the mRNA level is inducible by cold or salt stress[32]. However, overexpression of *OsCDPK7* enhanced salt- and drought-induced, but not cold-induced, expression of target genes, suggesting that the effect of this CDPK is specific to salt and drought signalling.

Another group of proteins identified as interacting with Ca^{2+} to effect an end response include serine/threonine phosphatases[33] (PPases). The type-2C PPases include a group of proteins with similarities to the calcium sensor protein calcineurin B (Refs 34,35) that are referred to in *Arabidopsis* as calcineurin-B-like (AtCBL) proteins. One of these, SOS3, is involved specifically in salt stress tolerance[34] and the gene encoding another, *AtCBL1* (but not those of other family members), is highly upregulated by cold and drought. Specificity through AtCBL isoforms might result from variation in the N-terminal sequence of AtCBL proteins, meaning that only some members have the potential to be targeted to membrane sites[36].

As well as achieving specificity through the use of different isoforms, calcium sensors might also serve as nodes at which cross-talk can occur. $[Ca^{2+}]_{cyt}$ elevations

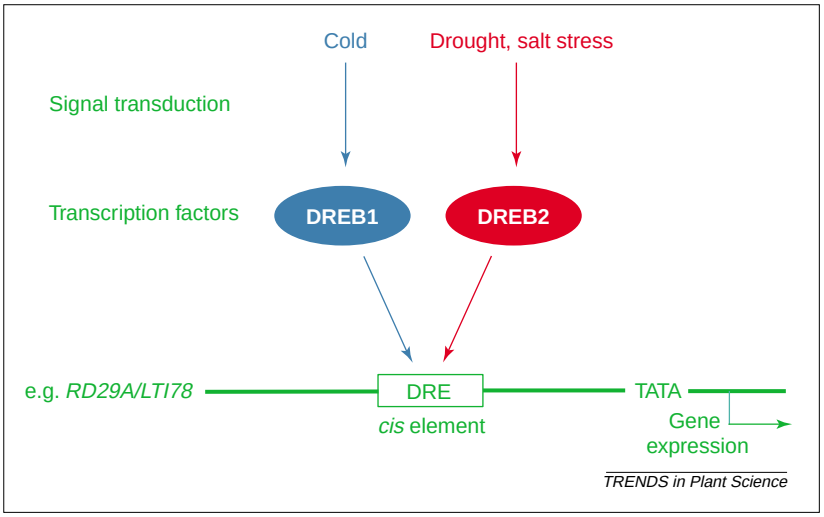

Fig. 2. The DREB1 and DREB2 transcription factors, key components in cross-talk between cold and drought signalling in *Arabidopsis*. Cold and drought activate the expression of the DREB1 and DREB2 families of drought-responsive-element-binding (DRE-binding) transcription factors, respectively. Both sets of transcription factors alight on the same *cis*-acting element in the promoters of genes such as *RD29A* (also known as *LTI78*) called the DRE element. Therefore, the DRE element is an integration point for cross-talk between cold and drought signalling in *Arabidopsis*. For simplicity, the abscisic-acid-responsive element is not shown on this diagram.

elicited by a specific stress could regulate phosphatases and kinases involved in the transduction of another stimulus. In alfalfa cells, cold-induced inactivation of protein phosphatase 2A (PP2A) is controlled by Ca^{2+} influx[37], but it is conceivable that PP2A activity could also be modulated via other stress-induced $[Ca^{2+}]_{cyt}$ elevations.

The ABI1 and ABI2 proteins (identified through the *abi1* and *abi2* mutations) are homologous to type-2C PPases[38,39] and have been implicated as negative regulators in the early stages of ABA signal transduction[40,41]. These proteins are potential nodes for cross-talk between different signalling pathways involving ABA (e.g. cold and drought). Recently, a tobacco homologue of the *Arabidopsis PP2C* genes has been identified, *NtPP2C1*, transcript accumulation of which is upregulated by drought treatment but inhibited by oxidative stress or heat[42]. If NtPP2C1 functions as a negative regulator in a similar way to *Arabidopsis* ABI1 and ABI2, it is possible that downregulation of its expression by oxidative and heat stresses could increase cellular sensitivity to ABA during drought treatment.

MAPK cascades

MAPK cascades are activated by numerous abiotic stresses[43] but they can introduce specificity into the system. A MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK), which in turn phosphorylates a MAPK. Three major types of MAPKKK have been identified in *Arabidopsis*: CTR1, ANP1-3 and the AtMEKK class. *AtMEKK1* (Ref. 44) is expressed in response to abiotic stresses including cold, drought and mechanical stimulation. Of six target MAPKs, only two (AtMPK3 and AtMPK6) showed evidence of being phosphorylated in response to the addition of a constitutively active ANP1 (via endogenous MAPKKs)[45]. Hydrogen peroxide (oxidative stress) increased the activation of MPK3 above the level achieved with the constitutively active ANP1. Further experiments showed that active ANP1 could activate hydrogen-peroxide-inducible promoters but not drought- or cold- or ABA-responsive promoters, implying primary signal-specificity encoded within ANP1.

Reconstruction of the AtMEKK1 kinase cascade in yeast indicates that there is selectivity in the partners that associate with it. Using a combination of two-hybrid and yeast mutant complementation, it was found that AtMEKK1 preferentially associated with and activated the closely related MAPKKs, AtMKK2 and MEK1, which, in turn, associated with and activated the MAPK AtMPK4 (Refs 46,47). Thus, specificity could be encoded by only allowing certain MAPKKK–MAPKK–MAPK combinations in complexes, like 'chords' in a piece of music (H. Hirt, pers. commun.). There is evidence of cross-talk between these 'chords' during abiotic stress, such as between MAPK cascades leading separately to ATMPK4 and ATMPK6 (Ref. 48).

Transcription factors

Low positive temperatures increase the level of freezing tolerance in many plant species through cold acclimation[49], but this state can also be achieved in response to drought or by application of the phytohormone ABA (Ref. 50). Many genes that are induced by cold are also induced by drought or ABA (Ref. 51), probably because many cold-inducible genes encode proteins to protect the plant from the consequences of freezing stress, which include dehydration. The gene *RD29A* (also known as *LTI78* or *COR78*) has been used in several studies examining the convergence of these pathways. *RD29A* is one of many cold- and drought-regulated genes that have been found to contain the so-called DRE or CRT (drought-responsive or C-repeat element) in their promoters[52]. In *Arabidopsis*, two groups of transcription factors, DREB1 (also known as CBF) and DREB2, bind to this *cis*-acting element[4,51,53]. The *DREB1* and *DREB2* genes encode structurally different proteins and are induced specifically by low temperature and by salt or drought, respectively (Fig. 2). DREB2A and DREB2B are produced in the root only in response to salinity, but are produced in the stem and the root after drought treatment[54], offering a further level of specificity of response.

Overproduction of either DREB1 or DREB2 proteins in protoplasts increased expression of an artificial *RD29A*-promoter GUS fusion gene[4], indicating that the DRE promoter element is a point at which drought or salt and cold signal transduction pathways converge and that it can integrate information about these two stimuli (Fig. 2). It does seem strange that two entirely separate signalling pathways lead to the action of these two sets of transcription factors (Fig. 2) simply for them to result in the activation of the same *cis*-acting element in the

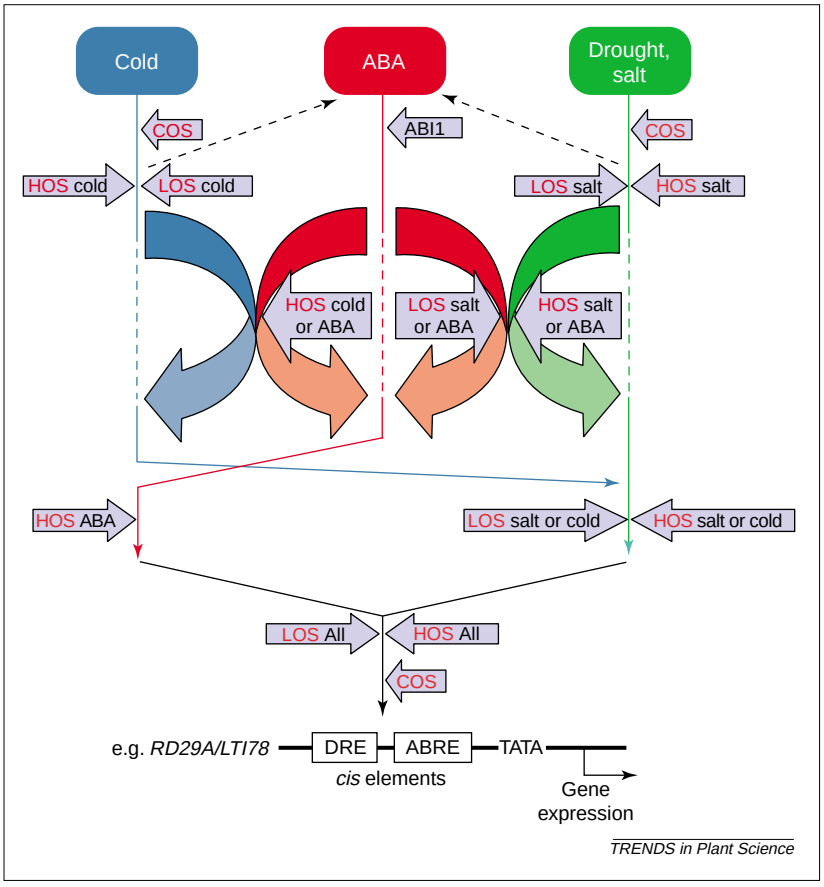

Fig. 3. Cold, osmotic stress and abscisic acid (ABA) signal transduction, as determined by the use of *los*, *cos* and *hos* mutants of *Arabidopsis*. The positions of HOS, COS and LOS gene products in the signalling pathways are indicated by pale blue arrows. The abscisic acid (ABA)-dependent pathway (red) interacts and eventually converges with ABA-independent pathways (dark-blue and green) to activate the expression of *RD29A* (also known as *LTI78*) and other genes containing the drought responsive and abscisic-acid-responsive promoter elements (DRE and ABRE) (black). Broken arrows indicate pathways (or parts of pathways) that cannot directly induce the expression of these genes but that require interaction with other pathways or branches of pathways. (Modified from Ref. 58.)

same genes. It might well be that, in other species, the DREB1 and DREB2 factors control the expression of two different sets of genes and that *Arabidopsis* has, during evolution, rationalized these two functions.

An ABA-responsive promoter element (ABRE) has been identified and a family of basic leucine zipper (bZIP) DNA-binding protein interact with it[55,56]. Genes such as *RD29A* contain both DRE and ABRE elements in their promoters and can be activated by ABA-dependent and ABA-independent pathways. A single ABRE element cannot function independently[56], for instance, *RD29B* has two copies. *RD29A* has both an ABRE and a DRE, suggesting that, in promoters containing both elements, the ABRE requires the DRE for ABA-induced expression. This suggestion is supported by data from the *sfr6* cold acclimation mutant of *Arabidopsis*[57], which is deficient in the expression of cold-regulated genes that contain a DRE element but expresses non-DRE-regulated cold genes normally. Interestingly, in *sfr6*, DRE-containing genes also failed to be expressed fully in response to stimulation by ABA, suggesting that there is cross-talk between the ABRE and DRE.

The interactions between these different pathways have been investigated in *Arabidopsis* through the analysis of mutants defective in the induction of *RD29A* by salt, cold, ABA or a combination of these[58]. The *HOS1* and *HOS2* loci encode signalling components that negatively regulate gene expression in response specifically to cold and not to other stress stimuli[59,60]. Conversely, HOS5 reduces expression in response to ABA and osmotic stresses but not to cold[61]. Combined cold and ABA or salt and ABA treatments have been shown to have a synergistic effect on *RD29A–LUC* expression[62], in contrast with the reduced levels of expression in response to combined cold and salt treatment. The results of these studies have suggested that ABA-dependent and ABA-independent osmotic and cold stress pathways might converge at several hitherto unexpected points (Fig. 3). If they do, this increases opportunities for coordination between stress signals and ABA in the regulation of gene expression.

Perspectives

Studying abiotic stress signalling pathways in isolation is valuable but it can be misleading because they form part of complex networks. In future, the onus will be on taking this fact into account, both intellectually and in terms of technology development. A perfect example of this is the availability of microarray technology. This enables researchers to examine the expression of not only all their particular stress-induced genes of interest but also thousands of others, without prejudice and without extra effort[63]. This will lead to a greater understanding of the effect that abiotic stress pathways have on each other as well as on pathways and processes that were not known to be connected.

The recent completion of the *Arabidopsis* genome project means that identifying the genes involved in specificity and cross-talk will be more rapid. This will then lead into work on the proteins themselves, to ask how exactly these proteins operate to encode specificity or to act as communicators or nodes in cross-talk. Finally, signalling components whose genes are induced by abiotic stress, and hence implied in abiotic stress signalling, can be directly tested for specificity or cross-talk (problems of redundancy aside) by phenotypic analysis of knockout mutants.

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