Signal transduction leading to low-temperature tolerance in *Arabidopsis thaliana*

Marc R. Knight

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
(marc.knight@plant-sciences.oxford.co.uk)

Calcium is used by most cells to convert external signals into biochemical events within the cytosol. To detect the effects of cold stress, a gene encoding apoequorin has been introduced into various cell types that, in the presence of coelenterazine, allows calcium levels to be monitored by the emission of blue light. All cell types respond to the cold by elevating calcium. This event is rapid after cold stress but then shows a slower kinetic response. The magnitude is dependent on both the rate and the final temperature to which cooling occurs. It would appear that calcium is transferred both from outside the cell and from the vacuole into the cytosol. The more rapid phase involves the former and the slower phase the latter. By studying mutant plants, it has been identified that an increase in intracellular calcium can activate the expression of transcription factors that control the subsequent transcription of a whole battery of genes which must be switched on to provide cold tolerance in the plant.

Keywords: calcium; signalling; acclimation; cold; *Arabidopsis*

1. INTRODUCTION

Calcium is used by most cells to convert external signals into cytosolic information, which can drive processes that are required for the full response to a particular stimulus. A rapid event following a low-temperature stimulus in plants is an elevation in the concentration of calcium in the cytosol: this appears to be linked very closely in time, and presumably mechanism, with the perception event. This presentation will introduce the role of calcium in the signal transduction pathway that leads to expression of cold-regulated genes in plants, particularly in *Arabidopsis*. There is a subset of plant cold genes whose transcription is regulated by a family of transcription factors, known as CBF or DREB1. Evidence will be presented for a role of calcium in mediating the expression of these CBF-responsive genes acting directly through the CBF transcription factors. An unexpected recent finding is that calmodulin, a major calcium-sensing protein that converts calcium signals into a usable response, actually seems to negatively regulate this pathway. Finally, the role of SFR6 in regulating CBF-mediated responses will be discussed. It should be emphasized that these low-temperature-signal transduction pathways have relevance to both chilling and freezing stress tolerance. The sequence of action of these genes is shown schematically in figure 1.

2. THE CALCIUM RESPONSE TO COLD

In order to measure cytosolic calcium levels non-invasively, the gene encoding apoequorin, a protein found in the jellyfish *Aequorea victoria*, has been introduced into

Arabidopsis. In the presence of coelenterazine, a functional protein aequorin is formed, and when two of the three highly specific calcium-binding domains of aequorin are filled, bound coelenterazine is oxidized, coelenteramide is then produced and blue light is emitted. This luminescence can be used to report on alterations in calcium concentration in the compartment where aequorin is located. Using this system, rapid (seconds to minutes) elevations in cytosolic calcium have been demonstrated in all vegetative tissues of both *Arabidopsis* and tobacco.

It seems the most important parameter that the plant senses to generate the cold-induced calcium elevation is the rate of cooling, as opposed to the absolute temperature. Plants challenged with more rapid cooling regimes show an increased magnitude of calcium response, and the relationship between cooling rate and magnitude of calcium elevation is approximately linear. When plants are challenged successively with identical cooling regimes, the calcium response becomes attenuated, rather like attenuation or accommodation in the nervous system; i.e. at a certain point the plant is no longer sensitive to the cold in terms of generating a calcium signal. That desensitization can be overcome simply by reducing the temperature. Therefore, although the rate of cooling is the most important parameter, the absolute temperature does have the ability to sensitize the system. If the temperature is reduced from 20 °C down to 10 °C or from 10 °C to 0 °C at the same rate of cooling, the response at 10–0 °C is higher than the one at 20–10 °C.

Where is the calcium coming from? We know from using inhibitors that the majority of the calcium comes from outside the cell. By targeting the calcium reporter to different parts of the cell, for instance to the cytosolic face of the vacuolar membrane, release of calcium from the vacuole can be measured. The calcium response to low temperature is biphasic, with a rapid spike followed by a

One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

Figure 1. Model for calcium regulation of *cor* gene expression.

smaller but more prolonged elevation. The slower the cooling rate, the more pronounced is the second peak. When plants with aequorin, which is targeted either to the cytosol or to the tonoplast, are compared, it appears that the second calcium elevation occurs by release from the tonoplast. We therefore think there are two sources of calcium, one coming from outside the cell into the cytosol, and one being released from the vacuole. The vacuolar release appears to be mediated by inositol triphosphate through phospholipase C.

Which cells are responding to cold to generate a calcium signal? Although whole plant imaging suggests that the calcium elevation is ubiquitous, these images are two-dimensional and do not yield information on cell or cell layer specificity. By expressing the calcium reporter only in specific cell types, the cell specificity of cold-induced calcium transients can be studied. This is not just a descriptive exercise, because different parts of plants will have different problems in response to low temperature and will need to respond differently, promoting or repressing gene expression in a cell-specific manner. Fusion of the aequorin protein to yellow fluorescent protein and by using enhancer-trap technology in collaboration with a group in Cambridge, expression of aequorin in different cell types was achieved.

The roots of *Arabidopsis* are very simple, with four layers of cells: the epidermis, cortex, endodermis and pericycle. Aequorin was targeted to each of those cell layers individually. This demonstrated that, although each of those cell types responds to cold with a calcium elevation, there are subtle differences in the magnitude of the responses. For instance the pericycle, which is buried deep within the tissue, gives the greatest calcium elevation in response to low temperature and the least in response to osmotic shock. At a more detailed level, using the chameleon's fluorescent calcium reporter system from Julian Schroeder

and Gethyn Allen in California, we found that all the epidermal cell types, including stomatal guard cells, epidermal cells and trichomes, responded to cold with an elevation in cytosolic calcium. We have not yet found a cell type in the plant that does not respond to cold by elevating calcium.

3. CALCIUM REGULATION OF GENE EXPRESSION

What does calcium actually do? Calcium is a ubiquitous internal signal, which activates various processes in the cell. Clearly, it activates the expression of genes whose products are required for stress tolerance. For example, the transcript levels of *kin1* are increased after less than 1 h, but if the calcium influx triggered by low temperature is inhibited by channel blockers, such as lanthanum, the cold-induced increase in *kin1* transcript abundance is reduced. It seems quite apparent that calcium is required by plants to activate important genes required for stress tolerance.

Because inhibitors present problems of specificity, we were keen to find genetic approaches to confirm or refute the possibility that calcium is actually required for gene expression and for stress tolerance. We have been studying mutant plants shown by other groups as having physiological problems with coping with low temperature, such as the *sfr* mutants described by Gary Warren. We have simply asked the question 'how many of these mutants are aberrant in their ability to deal with low temperature because they have a problem with signalling?' We measured the expression of cold-regulated genes and selected those mutants that have reduced *cor* gene expression. We then analysed the calcium signature of these plants in response to cold to determine whether the altered gene expression is related to aberrant calcium elevation.

The *chs3* chilling-sensitive mutant of *Arabidopsis* is indistinguishable from the wild-type when grown at 20 °C, but when challenged at 12 °C the lack of tolerance to low temperature manifests itself in yellowing and, eventually, death. This mutant was screened for cold-induced expression of *LTI78* and *KIN1*, genes that have the CBF-binding element within their promoters, and are known to be calcium regulated. If these plants are challenged with cold for 1, 2 and 3 h the normal wild-type plant will express these genes but the *chs3* mutant does so to a lower level. We thought it was possible that the mutant might have an aberration in signalling. We therefore measured cold-triggered calcium responses and found something rather interesting. When challenged between 10 °C and 0 °C there is very little difference between the wild-type and mutant calcium response; however, between 20 °C and 10 °C the *chs3* mutant has a very small calcium signal. Between 15 °C and 12 °C is the temperature range where these plants are struggling physiologically. This is only a correlation, but it comes from a different type of approach: a correlation between a reduced calcium response, a reduced level of gene expression, and reduced tolerance. We are moderately confident from the data obtained that calcium is required for activation of these genes and for stress tolerance.

We have been focusing on a set of cold-responsive (*cor*) genes that have a promoter element, CRT. A small family of cold-induced transcription factors, known as CBF or

DREB1, bind to the CRT element and promote transcription of these *cor* genes. Interestingly, transitional transcript levels of the CBF transcription factors are reduced in the *chs3* mutant. This gave us an insight into how calcium might be regulating the expression of the *cor* genes. If a reduced calcium response in this mutant leads to an effect on the transcription factor expression, then this would explain why expression of genes that rely on this transcription factor is not activated. So we asked the question: 'Does calcium regulate these genes through these specific transcription factors and through the binding site of the promoter that this transcription factor recognizes?' To answer this question, we made an artificial gene with the promoter element, CRT, as a concatamer fused to a reporter gene. The only way in which this gene can be activated is if this CRT element is activated through one of the CBF/DREB transcription factors. We have demonstrated that the artificial gene acts as a cold-regulated gene, and is induced when plants are exposed to low temperature.

The next step was to determine whether calcium regulates this artificial gene. If it does, that would suggest that calcium is acting specifically through the CRT element in the promoter and specifically through transcription factors which bind these. We elevated calcium artificially by treatment with mastoparan, a toxin from a wasp that activates G proteins and produces a calcium elevation. Under ambient temperature, 10 and 20 μ M mastoparan induced expression of the artificial reporter gene. This induction by mastoparan at ambient temperature could be inhibited by chelation of calcium with ethylene glycol bis-2-aminoethyl ether-*N,N'*,*N''*,*N'*-tetraacetic acid. So simply increasing calcium will induce the expression of this artificial gene and would indicate that calcium can activate this specific promoter element.

We wanted to see whether calcium could regulate the expression of the CBF transcription factors that bind to the CRT element. We did this experiment in a different way. If calcium is added to plants as they are being cooled, the characteristic calcium spike is bigger because of the increased calcium gradient. This enables comparison of expression patterns under different calcium transients but identical cooling regimes. When subjected to a typical cold treatment, the CBF transcription factors are induced within 1 h, peak at 2 h and have disappeared at 6 h. However, with the enhanced calcium response, the expression of CBF is much higher and is prolonged for up to, or in excess of, 6 h. This suggests that calcium is a positive factor activating expression of the CBF transcription factors.

To summarize, low-temperature treatment results in an elevation in the cytosolic concentration of calcium and this seems to be ubiquitous in all cell types and, in fact, all plant species we have looked at. This includes both chilling-sensitive plants like tobacco and cold-acclimatable plants like *Arabidopsis*. If there are commonalities between signalling in chilling-tolerant and chilling-sensitive plants and cold-acclimatable plants and non-cold-acclimatable plants, then one of the common elements is likely to be calcium. It may be that the end response is different, but calcium is definitely ubiquitous. Calcium seems to regulate the expression of the CBF genes that control transcription of a whole battery of genes that are required for tolerance.

What is used by the cell to convert the calcium signal in the cell into these useful responses? Although there are a number of possibilities, the most obvious candidates in the plant world are calmodulin, which is the most commonly cited mediator of calcium-dependent processes in any eukaryotic cell, and CDPKs. The CDPKs, a unique form of protein kinase found only in plants, are hybrid proteins with a calmodulin-like domain and a kinase-like domain. Although there is little hard evidence, the literature is full of speculation that these two are involved in cold signalling. We are investigating both calmodulin and CDPKs but present only preliminary data on calmodulin. We have made a construct consisting of calmodulin under the control of a steroid-inducible promoter. Northern analysis shows that, when transgenic plants are treated with steroids, there is a huge expression of calmodulin at 20 °C and 4 °C that does not occur in the absence of steroids. When transgenic plants are treated with steroids to increase calmodulin levels, cold-induced expression of the *cor* genes *KIN1* and *LTI78* is significantly reduced. This reduction in cold-induced *cor* gene expression does not occur if the transgenic plants are not treated with steroids. Equally, when control plants, which did have the calmodulin construct present, were treated with steroids, no effect on cold-induced *cor* gene expression could be detected: i.e. the effect on *cor* gene expression is genuinely dependent on the levels of calmodulin and is not an artefact of the methodology.

We were expecting calmodulin to act as a positive regulator that would actually enhance calcium-mediated *cor* gene expression, but what we found was the reverse. It seems calmodulin is negatively regulating the expression of these genes and, in fact, we have a number of transgenics which show different levels of calmodulin expression, and there is an inverse relationship between calmodulin levels and *cor* gene expression. Although plants may not actually use calmodulin to negatively regulate *cor* gene expression, it appears they have this potential. This leaves calcium-dependent kinases on the menu as positive regulators. Why calmodulin should be a negative regulator is another question. There are other processes in plants where calmodulin and calcium-dependent kinases, although both activated by calcium, have antagonistic roles: one activates while the other deactivates. It could be that calmodulin is involved in the reduction of the response when it needs to be terminated. Often gene expression is required to be transient and it may be that CDPKs may mediate the calcium-dependent activation of the genes whereas calmodulin's role is to subsequently bring the response down.

Finally, the principle by which plants are pre-treated for cold acclimation is well known. Gary Warren identified a number of mutants that are unable to cold acclimate and we were interested whether these might be signalling mutants. Studies of *cor* gene expression carried out on these mutants demonstrated that one of them, *sfr6*, showed no, or very low levels of, cold-induced expression of these genes, even after 48 h of exposure to low temperature. We found that there was no difference whatsoever between *sfr6* and the wild-type in terms of calcium response, so whatever is wrong with *sfr6*, it is not calcium signalling. This suggests that the lesion must occur either downstream of calcium or independently of calcium.

What was interesting is that the CBF transcription factor that promotes expression of these *cor* genes was expressed normally. The *CBF* genes do not have CRT elements within their promoters and do not autoregulate themselves. This gave us a clue that maybe *sfr6* cold-induced gene expression was only aberrant in the *cor* genes that were dependent on CBF for cold-induced expression. Therefore, we looked at the expression of *ATP5CS1*, another gene which is cold regulated but does not contain the CRT element. We found that cold-induced expression of *ATP5CS1*, like the CBF transcription factors, is perfectly normal *sfr6*. We propose that *sfr6* mediates the interaction between CBF and CRT.

We have tested this directly by using an artificial gene consisting of the CRT element fused to a reporter gene, luciferase. The reporter gene will only be expressed if CBF binds to the CRT element. We analysed luciferase expression in the wild-type and *sfr6* segregants. Expression of the reporter gene is much lower in *sfr6*. Clearly, *sfr6* cannot activate the CRT element in response to cold or, in fact, to drought. In collaboration with David Galbraith in Arizona, we performed some microarray analysis of *sfr6*. Gary Warren worked with the promoters of 50 genes that were downregulated in *sfr6*. We were hoping that they would all contain the CRT element, but 6% contained a copy of the eight CRT nucleotide motif in its purest form, 16% the motif with one mismatch, 32% the element with two mismatches and 38% had only the core of the element, CCAGC. This means one of two things: either *sfr6* also affects other genes that have different elements, or the other genes that are affected require the expression of CRT-containing genes. These are the only logical possibilities.

The CRT element, with which we have been working, can also be activated by drought but by a completely different set of transcription factors called DREB2. The DREB2 transcriptional activators are very different in sequence and structure from the CBF family; drought can only activate the CRT element through DREB2s, and cold can only activate it through CBF. The *sfr6* mutants cannot activate these genes in response to either drought or cold. This implies that *sfr6* has some function that is required for the activity of both of these sets of transcription factors.

I thank the people who did this work. Heather Knight did all the work on gene expression presented here. Work with the mutants was done in collaboration with Gary Warren's group in London. Christoph Plieth performed the work on cooling rates and explained the fundamental parameters required for calcium signalling. Helen Townley did the work on calmodulin. Andrew Parsons worked with the cell-specific calcium signalling in the guard cells, and Cathy Moore studied calcium responses in root cells in collaboration with Mark Tester, Jim Haseloff and Ed Kiegle. I also thank Maggie Smallwood for compiling this.

Discussion

M. Kutozov (*School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, UK*). Have you tried other calmodulin isoforms? There are at least eight of them, and I think it is possible that when calmodulin 3 is overexpressed, it may override another isoform which may be a positive regulator.

M. R. Knight. So what you are saying is that overexpression of the calmodulin 3 might actually act as a dominant negative. By overexpressing calmodulin 3 we are interfering with another calmodulin, which is a positive regulator.

M. Kutozov. There might be another isoform which would be specific, therefore its target is a positive regulator.

M. R. Knight. It is possible. We chose calmodulin 3 specifically because it is the one that is most highly expressed in response to cold. We assumed that it must be involved, but other isoforms could be present. We have probed for calmodulin 1, which is expressed, and it does not have an effect on gene expression. It could have an effect on the activity of the calmodulin, implying that calmodulin 3 cannot independently activate. This would require that the differences in protein structure between the calmodulins are highly significant in terms of their function.

M. Kutozov. I think there was at least one report that the different isoforms may differ functionally.

M. R. Knight. Yes. There is work in the soya bean with pathogeneses where they have expressed those three.

M. A. Marahiel (*Department of Chemistry, Philipps-Universität Marburg, Marburg, Germany*). I suggest that a primary factor signals calcium induction. What may be coming through the membrane is a hormonal action. Some examples of hormonal receptor interaction with calcium are known. What is the first signal that leads to cold sensing through the membrane?

M. R. Knight. There are a lot of candidates. It could be a direct activation of calcium channels by cold via the mechanical properties of the membranes. If it is simply a stress-activated channel then it could be activated by this route. It could be voltage activated. There could be depolarization caused by potassium. As Professor Dianna Bowles said, the candidates upstream of cold sensing are membrane fluidity and the cytoskeleton. In the literature, both of those are reported to affect calcium signalling. My feeling is that it is to do with stretch activation, and that altering the physical properties of the membrane causes stretch activation.

M. A. Marahiel. Are there candidates in the membrane for that?

M. R. Knight. The *Arabidopsis* genome has only two candidates for homologues to calcium channels and they are not of the type that you would expect. If you do any homology searching in the genomes of *Arabidopsis* you do not find the classical channels. Experimentally, there are mechanically activated channels in onion cells, which respond to cold. My hope is that mutants will identify something further. We can modulate membrane fluidity and cytoskeleton and affect calcium signalling, but we do not really know details of the mechanism.

D. K. Hincha (*Max-Planck-Institute für Molekulare Pflanzphysiologie, Potsdam, Germany*). If overexpression of calmodulin reduces *cor* gene expression, is the reverse also true? Have you tried to antisense the calmodulin?

M. R. Knight. No, what we have done is expressed calmodulin inhibitory peptides. This provides enhancement of the *cor* gene expression. It would point towards a general inhibition of calmodulin.

P. L. Davies (*Department of Biochemistry, Queen's*

University, Ontario, Canada). You mentioned calmodulin and kinases as mediators of the calcium signal. I was just wondering if you considered proteases like the calpains?

M. R. Knight. No we have not. Any calcium-dependent protein that is capable of post-transcriptional modification is a candidate, because we do not know how the transcription factors are regulated. Calmodulin kinase and others, such as some calcium phosphatases, could be examined. Are the calpains seen to be important in cold signalling in other organisms?

P. L. Davies. I am familiar with them in mammalian cells and they are involved in calcium signalling but certainly not in response to cold.

M. R. Knight. No, but in regulation of gene expression?

P. L. Davies. Yes.

P. Quick (*Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK*). The calcium activates many processes and the shape of the signal has often been thought to be just as important as the amplitude. Do you have any evidence for different types of shape?

M. R. Knight. Different types of calcium signals, signatures?

P. Quick. Is there a specific cold signature of calcium?

M. R. Knight. Yes. Because calcium is ubiquitous, you can have a list as long as your arm of processes which it can control. Pretty much every abiotic stress in plants causes an elevation in calcium. Yet, each of those stresses can cause a specific response. If calcium mediates specificity, each one must be diagnostically different so the cell can tell them apart; yes, you can diagnose a response. If you show me a calcium response, I will tell you what the plant is responding to. Is there evidence that calcium is used by the plant to convey specific information? I would have to say the answer is no and I will give you empirical data which support this.

If you elevate calcium, using things like mastaperan and

various other calcium agonists, you do not generate anything like the profile of cold. So cold is very specific in terms of its distribution between vacuole and external release. There is a suggestion that information is coded in such signatures. We are looking downstream of calcium because there is likely to be specificity in the proteins that decode that information. They may be present only in certain cells that are responsive to specific stimuli in specific locations.

I think it is an open question, but yes, the cold response is different from anything else.

J. Laybourn-Parry (*School of Life and Environmental Sciences, University of Nottingham, Nottingham, UK*). It was very elegant that you had your reporter gene in specific cell types. Is there any evidence that any of those cell types do respond differently to cold in those early signalling events?

M. R. Knight. Yes. For instance, the endodermal cells show oscillations whereas the other cells do not. There are magnitude differences but whether they are significant is not known.

We have a problem because we are just looking at gene expression. The genes which are calcium regulators are not expressed everywhere, whereas the calcium signal is expressed everywhere. There appear to be situations where the calcium gate is not open, yet the calcium is elevated. So presumably, in those cells, calcium is regulated by some other process.

GLOSSARY

CaM: calmodulin

CBF: C-repeat element binding factor

CDPK: calcium-dependent kinase

CRT: C-repeat element

DRE: drought-responsive element

DREB: drought-responsive element binding protein