

Cold-activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca²⁺ influx

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Summary

Previous studies on cold-triggered events leading to Ca²⁺ influx during cold acclimatization have been conducted on either unicellular cyanobacterium *Synechocystis* or plant cell suspensions, and used transcript levels of cold-induced genes as end-point markers. Whether the results of these studies are valid for intact plants or their organs is not known. Here we examine cold signaling in transgenic *Brassica napus* seedlings carrying, in addition to the endogenous cold-inducible *BN115* gene, the β -glucuronidase (GUS) gene placed under control of the *BN115* promoter. The activity of *BN115* promoter was monitored at the transcriptional and translational levels by determining accumulation of *BN115* transcripts and by histochemical assay of GUS activity. Cold-activation of *BN115* was strongly inhibited by the membrane fluidizer benzyl alcohol, but mimicked at 25°C by the membrane rigidifier dimethylsulfoxide (DMSO). The cold induction of *BN115* was also inhibited by stabilizers of microtubules and actin microfilaments, taxol and jasplakinolide, respectively, but was mimicked at 25°C by microtubule destabilizer oryzalin or colchicine, or by microfilament destabilizer latrunculin B. Gd³⁺ or ruthenium red prevented the cold activation of *BN115*, but Ca²⁺ ionophore A23187 or cyclic ADP-ribose activated it at 25°C. Inhibitors of tyrosine kinases, protein kinase C and phosphoinositide kinases prevented the cold activation of *BN115*, but inhibitors of protein phosphatases (PP) 1 and 2 A activated *BN115* at 25°C. Constitutively expressed GUS activity in another transgenic line of the same cultivar of *B. napus*, was not affected by cold or any of the chemical treatments used in the experimentation. Activation of *BN115* at 25°C by DMSO, Ca²⁺ ionophore, cADPR, and by inhibitors of PP1 and 2A was accompanied by an increased freezing tolerance. It was concluded that the cold-activation of *BN115* requires membrane rigidification, cytoskeleton reorganization, Ca²⁺ influx and action of several types of protein kinases.

Keywords: cold sensing, membrane fluidity, cytoskeleton, calcium, gene activation, protein kinases, GUS reporter gene.

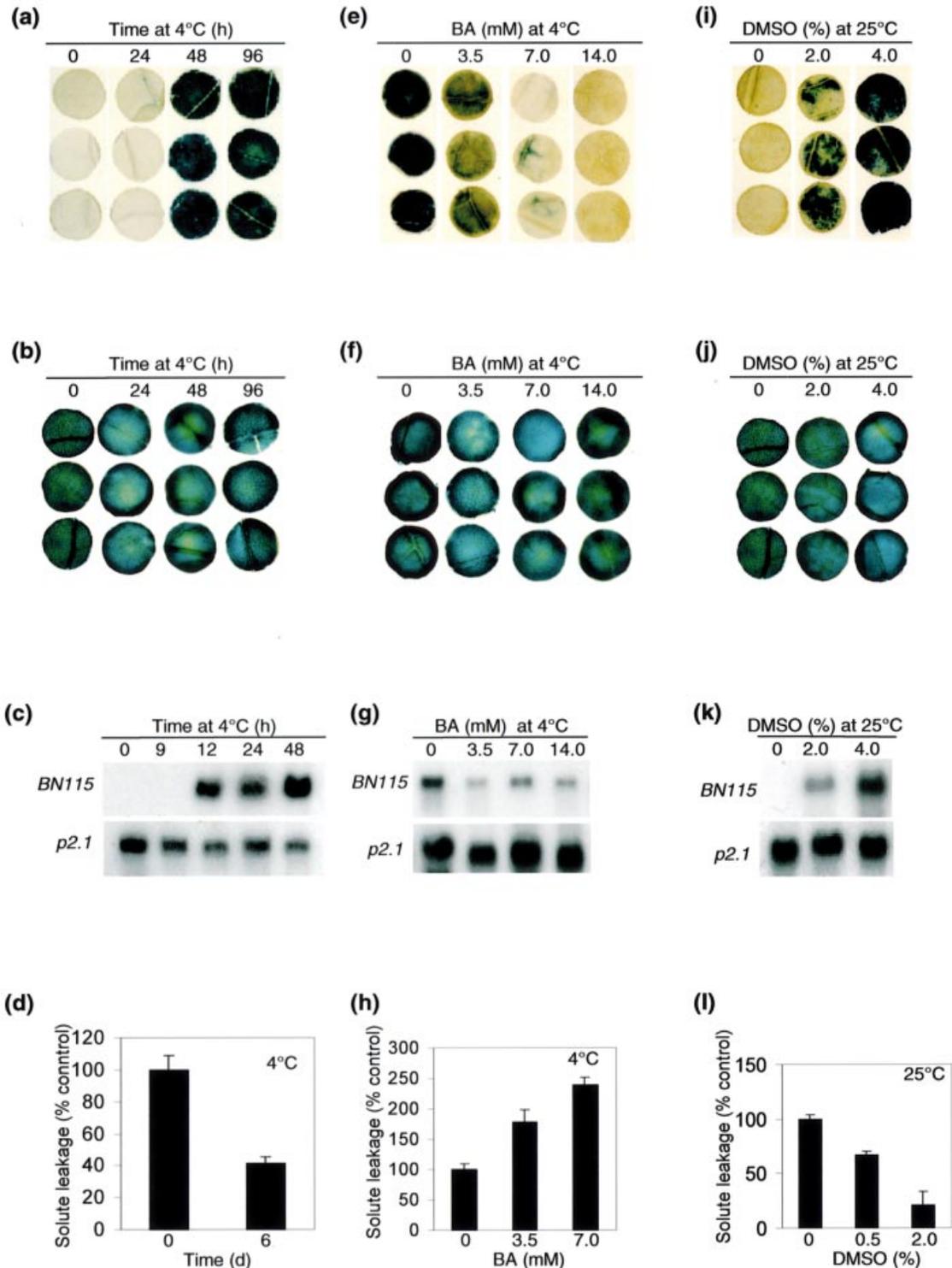
Introduction

Plants capable of cold acclimatization sense the low but non-freezing temperatures during the onset of winter and trigger specific biochemical processes that result in the acquisition of tolerance to subsequent freezing temperatures (Levitt, 1980). Cold-acclimatization is associated with novel gene expression, *de novo* protein synthesis and many physiological changes in the plant (Guy, 1990). A large number of cold-inducible genes have been identified and characterized in several plants (Thomashow, 1999),

including alfalfa (Mohapatra *et al.*, 1989; Monroy *et al.*, 1993a; Wolfrain *et al.*, 1993), *Arabidopsis* (Gilmour *et al.*, 1992; Nordin *et al.*, 1991; Yamaguchi-Shinozaki and Shinozaki, 1993), barley (Dunn *et al.*, 1991), *Brassica napus* (Weretilnyk *et al.*, 1993; White *et al.*, 1994) and wheat (Houde *et al.*, 1992). One of the cold-induced genes of *B. napus* is *BN115* (White *et al.*, 1994), an orthologue of the *Arabidopsis cor15* gene (Thomashow, 1999). The latter is known to encode a chloroplast-targeted

protein (Lin and Thomashow, 1992) and, when constitutively expressed, confers resistance to freeze-induced membrane damage (Artus *et al.*, 1996; Steponkus *et al.*, 1998). However, the upstream events leading to the cold-induction of this gene have not been examined.

A rapid and transient rise in cytosolic Ca²⁺ level is known to be triggered by cold (Knight *et al.*, 1991) and is required for cold-induced gene expression and cold acclimatization in alfalfa (Monroy *et al.*, 1993b) and *Arabidopsis* (Tahtiharju *et al.*, 1997). Most of the research on early



events upstream of Ca^{2+} influx and in relation to cold acclimatization, has focused on alfalfa cell suspension cultures (Dhindsa *et al.*, 1998) or on the unicellular cyanobacterium *Synechocystis* (Murata and Los, 1997; Nishida and Murata, 1996). Whether the results of these studies on unicellular systems are applicable to entire seedlings or their organs remains to be determined. Furthermore, previous studies have only monitored the accumulation of transcripts of end-point marker genes. However, the development of cold-induced freezing tolerance requires *de novo* protein synthesis (Chen *et al.*, 1983). Whether the cold signaling cascade leads to the marker gene activity at the translational level is not known.

Here we examine the nature of early events leading to the cold activation of *Brassica napus* gene, *BN115*. We used a transgenic line of *B. napus* cv. Westar containing, in addition to the endogenous cold-inducible *BN115* gene, the β -glucuronidase (*GUS*) gene placed under control of the *BN115* promoter and thus rendered cold-inducible. Another transgenic line of the same cultivar contained *GUS* gene placed under the control of the tobacco cryptic constitutive promoter *tCUP*. Thus the seedlings of this line expressed *GUS* activity constitutively as a control for the cold-inducible *BN115*-driven *GUS* activity. As end-point markers of the cold signaling process, we monitored the accumulation of the endogenous *BN115* transcripts, *BN115*-driven *GUS* activity, and development of freezing tolerance in *Brassica napus* leaves. The results of the present investigation show that in *B. napus* leaves (1) the cold activation of *BN115* promoter is inhibited by the membrane fluidizer benzyl alcohol (BA), stabilizers of microfilaments and microtubules, Ca^{2+} chelators and channel blockers, and by inhibitors of several specific protein kinases, but is mimicked at 25°C by the membrane rigidifier DMSO, destabilizers of microfilaments and microtubules, Ca^{2+} ionophore A23187, cADP-ribose and inhibitors of protein phosphatases (PP) 1 and 2 A; and (2) activation of *BN115* at 25°C by dimethylsulfoxide (DMSO), Ca^{2+} ionophore, cADPR or by inhibitors of protein

phosphatases (PP) 1 and 2A, is accompanied by development of freezing tolerance.

Results

Cold activation of the BN115 promoter-GUS activity in transgenic Brassica

The leaves of transgenic *Brassica napus* seedlings expressing *GUS* activity under the control of either the cold-inducible *Brassica napus* *BN115* promoter, or the tobacco cryptic constitutive promoter *tCUP*, were exposed to 4°C for different times. The leaf discs were then punched out for determining *GUS* activity as a measure of *BN115* activation. Leaves placed at 4°C exhibited high levels of *BN115* promoter activity. The time required for the detection of activity varied with the level of expression examined, that is translational or transcriptional. When measured in terms of *GUS* activity, *BN115* activation was hardly detectable at 24 h, but by 48 h it had reached maximum levels as indicated by the intensity of blue coloration of the leaf discs (Figure 1a). It should be noted that *GUS* activity expressed constitutively under the control of *tCUP* promoter was not affected by cold (Figure 1b). When *BN115* activity was measured in terms of accumulation of its endogenous transcripts, it could be detected at high levels at 12 h of cold treatment (Figure 1c). The leaves kept at 25°C (0-time) did not show any activity (Figure 1a,c). To measure freezing tolerance, cold-acclimatized (CA) and nonacclimatized (NA) leaves were subjected to solute leakage test. It was found that solute leakage from NA leaves was more than twice that from CA leaves (Figure 1d), suggesting that CA leaves were more freezing tolerant than the NA leaves. It may therefore be concluded that *BN115* promoter is able to confer cold-inducible expression on *GUS* activity, and that such activity may be used as a reliable marker to study the regulation of *BN115* activation. Since both *BN115* transcript levels and *GUS* activity were easily detected at 48 h of cold treatment, in all

Figure 1. Cold-activation of *BN115* and regulation by modulators of membrane fluidity.

(a–d) Cold activation of *BN115*. Leaves were exposed to 4°C for the times indicated. One half of the *BN115*-*GUS* leaf was used to perform *GUS* assays, while RNA was extracted from the other half. (a) time-course of cold-induced *GUS* activity in *BN115*-*GUS* seedlings; (b) *GUS* activity at 4°C in *tCUP*-*GUS* seedlings expressing *GUS* constitutively; (c) time-course of cold-induced accumulation of *BN115* transcripts (upper panel) and constitutively expressed *p2.1* transcripts used as a control (lower panel). (d) cold-induced development of freezing tolerance. Solute leakage, inversely related to freezing tolerance, was determined in nonacclimatized (NA) and 6-d cold-acclimatized (CA) leaves and is presented as percent of solute leakage in NA leaves. Error bars represent standard deviation of the mean of four replicates.

(e–h) Inhibition of cold activation of *BN115* by different concentrations of the membrane fluidizer, benzyl alcohol (BA). Leaves were treated with BA first at 25°C for 3 h and then at 4°C for 48 h. Effects of BA on cold-induced *GUS* activity (e) constitutively expressed *GUS* activity (f) cold-induced accumulation of *BN115* transcripts (g, upper panel), constitutively expressed *p2.1* transcripts (g, lower panel), and on cold-induced development of freezing tolerance (h). Solute leakage is presented as percent of that in the untreated control leaves. Error bars represent the standard deviation of the mean of four replicates.

(i–l) Activation of *BN115* by different concentrations of the membrane rigidifier dimethylsulfoxide (DMSO) at 25°C. Leaves were treated with DMSO for 6 h. Effects of DMSO on *BN115*-driven *GUS* activity (i) constitutively expressed *GUS* activity (j) accumulation of cold-inducible *BN115* (k, upper panel) and constitutively expressed *p2.1* (k, lower panel) transcripts, and on development of freezing tolerance (l). Solute leakage is presented as percent of that in untreated control leaves. Error bars represent the standard deviation of the mean of seven replicates. Each experiment was repeated at least 3 times and yielded similar results each time.

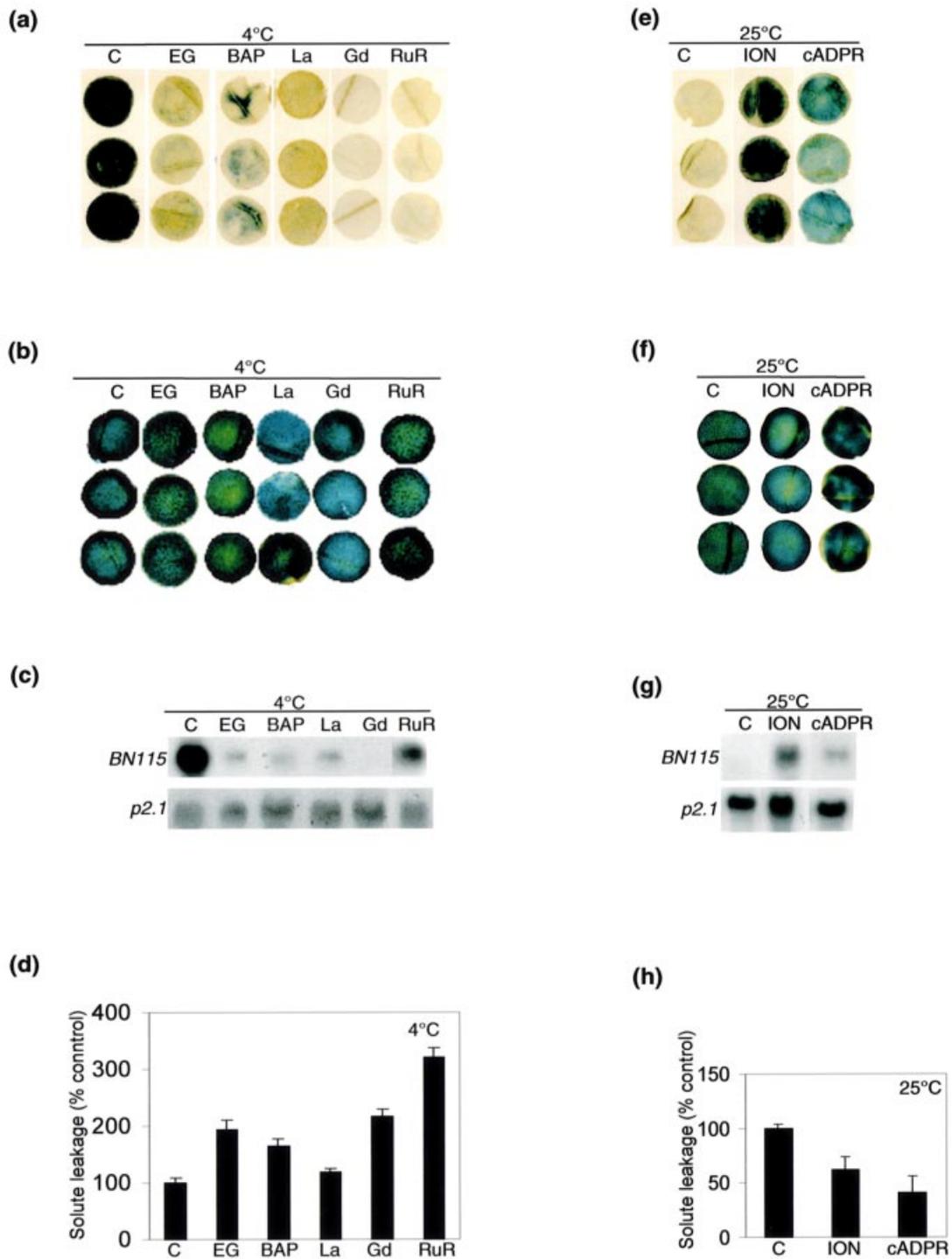


Figure 2. Cold-activation of BN115 requires Ca²⁺ influx.

(a–d) Leaves were treated at 25°C for 3 h with the Ca²⁺ chelators EGTA (EG, 33.33 mM) or BAPTA (BAP, 1.33 mM), or with the Ca²⁺ channel blockers, lanthanum chloride (La, 0.67 mM) or gadolinium chloride (Gd, 0.67 mM), or with ruthenium red (16.67 μM), a blocker of internal Ca²⁺ release, and then transferred to 4°C for 48 h. Effects of these inhibitors of Ca²⁺ availability at 4°C on cold-inducible (a) and constitutively expressed (b) GUS activity, accumulation of cold-inducible *BN115* transcripts (c, upper panel), constitutively expressed *p2.1* transcripts (c, lower panel), and on cold-induced freezing tolerance (d). Error bars represent the standard deviation of the mean of four replicates.

(e–h) Effects of 10 μM Ca²⁺ ionophore A23187 (ION) or 10 μM cADPR at 25°C on *BN115*-driven (e) or constitutive (f) GUS activity, accumulation of *BN115* transcripts (g), and on freezing tolerance (h). Solute leakage, an inverse measure of freezing tolerance, is presented as percent of that in untreated control leaves. Error bars represent the standard deviation of the mean of seven replicates. Each experiment was repeated at least 3 times and yielded similar results each time.

subsequent experiments this period of cold treatment was used when required.

Cold activation of BN115 requires membrane rigidification

Membrane fluidity is known to be directly and reversibly affected by temperature and cold-induced membrane rigidification is considered as the primary cold-sensing event (Murata and Los, 1997; Örvär *et al.*, 2000; Vigh *et al.*, 1993). Such membrane rigidification at low temperature can be prevented by chemical membrane fluidizers such as BA and mimicked at 25°C by treatment with the membrane rigidifier (DMSO) (Örvär *et al.* 2000) or by catalytic hydrogenation of membrane lipids (Vigh *et al.*, 1993). Thus we examined the effects of BA and DMSO on *BN115* activity. Leaves were treated with different concentrations of BA and exposed to 4°C. The data in Figure 1(e,f) show that increasing BA concentrations strongly inhibited the cold-inducible GUS activity (Figure 1e) but the constitutive GUS activity was not affected (Figure 1f). The cold-induced accumulation of *BN115* transcripts was also inhibited by BA (Figure 1g) and the BA-treated leaves showed considerably more solute leakage than the untreated control leaves (Figure 1h). Thus treatment of leaves with BA inhibits the cold-induced *BN115* activation and development of freezing tolerance.

Treatment of *B. napus* leaves with the membrane rigidifier DMSO at 25°C showed a marked increase in *BN115*-driven (Figure 1i), but not the constitutive (Figure 1j), GUS activity, and caused the accumulation of *BN115* transcripts (Figure 1k). The solute leakage decreased with increasing concentration of DMSO (Figure 1l) suggesting that DMSO treatment results in an increased freezing tolerance at 25°C.

Cold activation of BN115 requires Ca²⁺ influx from cell wall and internal stores

Calcium is a well-known secondary messenger in many signaling pathways in plants, including temperature signaling (Trewavas and Mahlo, 1998). Since the cold-triggered Ca²⁺ influx may occur from Ca²⁺-rich cell wall and/or from intracellular Ca²⁺ stores, modulators of Ca²⁺ availability from both these sources were used in the present study. To examine the role of cell wall Ca²⁺, leaves were treated with a Ca²⁺ chelator EGTA or 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA), or with a Ca²⁺ channel blocker La³⁺ or Gd³⁺, first at 25°C for 3 h and then at 4°C for 48 h. Treatment of leaves with each of these chemicals differentially decreased the cold-induced GUS activity (Figure 2a) but had no effect on the constitutive GUS activity (Figure 2b). These inhibitors of Ca²⁺ availability strongly inhibited the cold-induced accumulation of

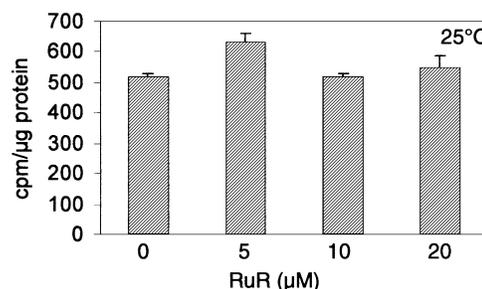


Figure 3. Ruthenium red does not affect rate of protein synthesis in *B. napus* leaves.

Leaves were treated with indicated concentrations of ruthenium red, and rate of protein synthesis was determined as described in experimental procedures. Error bars represent standard deviation from the mean of three replicates.

Experiments were repeated 3 times and yielded similar results each time.

endogenous *BN115* transcripts (Figure 2c). Gd³⁺, a specific blocker of mechanosensitive Ca²⁺ channels, was more effective than La³⁺ (a blocker of voltage-gated Ca²⁺ channels). The calcium inhibitors that reduced the cold-induced *BN115* activity also decreased the cold-induced development of freezing tolerance as reflected by the increased solute leakage (Figure 2d). Treatment of leaves with ruthenium red (RuR), known to block Ca²⁺ release from internal stores, abolished the cold-induced, but not the constitutive, GUS activity (Figure 2a,b) and caused a large decrease in the development of freezing tolerance as shown by the increased solute leakage (Figure 2d). The effects of RuR were less pronounced on the cold-induced *BN115* transcript accumulation (Figure 2c).

The effects of chemicals known to cause Ca²⁺ influx at 25°C were then examined. Treatment of leaves with the Ca²⁺ ionophore A23187, known to cause Ca²⁺ influx from the cell wall (Monroy and Dhindsa, 1995), or with cADPR which is known to release Ca²⁺ from internal stores (Allen *et al.*, 1995), resulted in *BN115* activation at 25°C. Thus A23187 or cADPR induced the *BN115*-driven (Figure 2e), but not the constitutive (Figure 2f) GUS activity. Both A23187 and cADPR caused the accumulation of *BN115* transcripts at 25°C (Figure 2g) and increased the freezing tolerance as indicated by the reduced solute leakage from leaves (Figure 2h).

Recently, ruthenium red (RuR) has been shown to inhibit protein synthesis in monkey kidney cells (Creppy *et al.*, 2000). Therefore, we examined the effects of RuR on the rate of protein synthesis in *B. napus* leaves. The effects of 0, 5, 10, and 20 μM RuR on the rate of incorporation of [³⁵S]Methionine into TCA-precipitable fraction were determined. The data presented in Figure 3 show RuR at a concentration up to 20 μM had no effect on the rate of protein synthesis. In experiments reported in Figure 2(a)–(d), 16.67 μM RuR was used. Therefore, at the concentration used in this study, RuR had no inhibitory effect on protein synthesis.

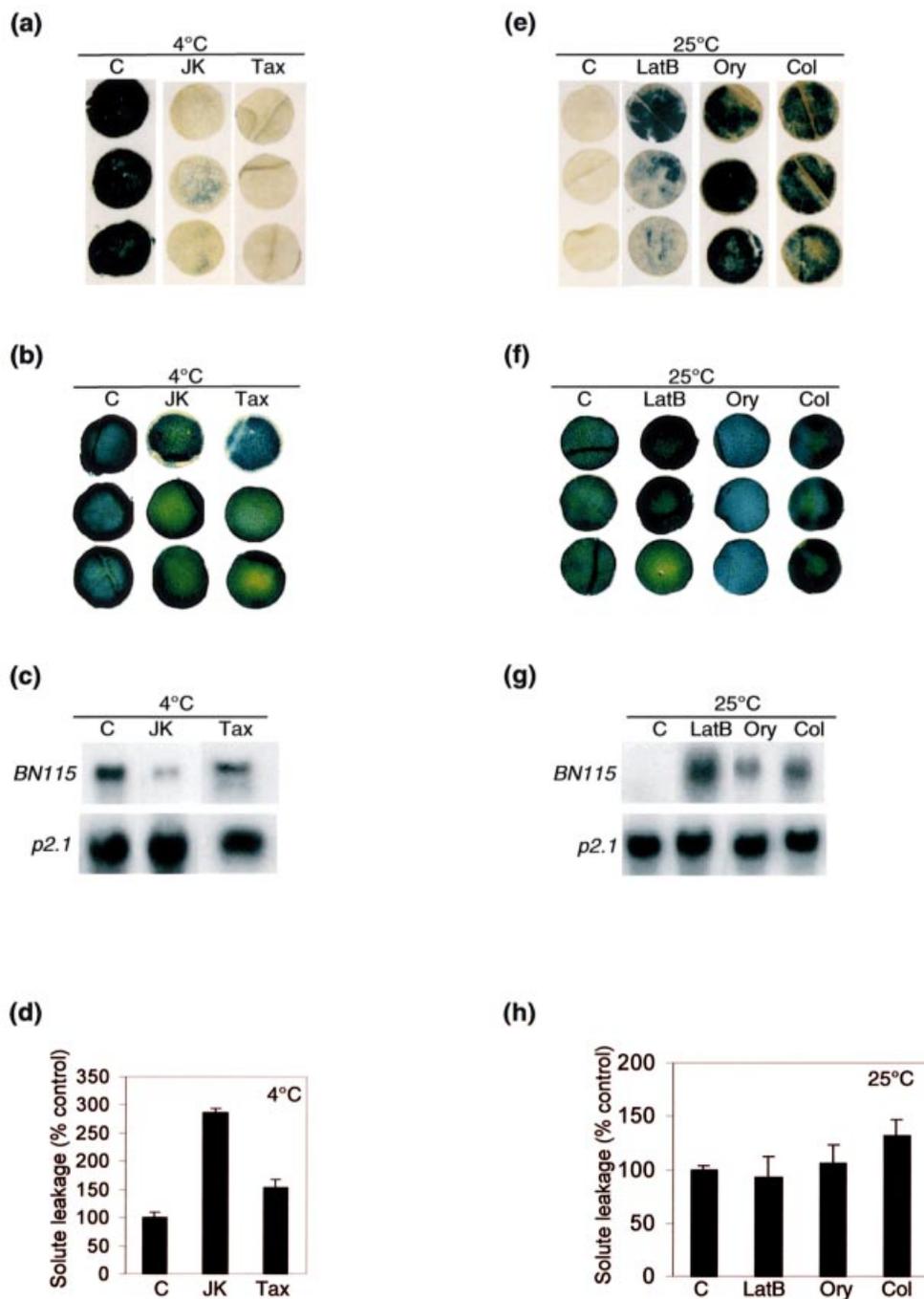


Figure 4. Cold-activation of BN115 requires reorganization of cytoskeleton.

(a–d) Leaves were treated with the microfilament stabilizer jasplakinolide (JK, 0.67 μM), or the microtubule stabilizer taxol (Tax, 50 μM) for 3 h at 25°C and then for 48 h at 4°C.

Effects of JK or Tax on cold-induced GUS activity (a), constitutively expressed GUS activity (b), accumulation of *BN115* transcripts (c) and development of freezing tolerance (d). Error bars represent the standard deviation of the mean of four replicates.

(e–h) Leaves were treated with the microfilament destabilizer latrunculin B (LatB, 20 μM), or the microtubule destabilizers oryzalin (Ory, 50 μM) or colchicine (Col, 400 μM) at 25°C for 6 h. Effects of LatB, Ory, or Col at 25°C on *BN115*-driven GUS activity (e), constitutively expressed GUS activity (f), accumulation of *BN115* transcripts (g), and freezing tolerance (h). Solute leakage, an inverse measure of freezing tolerance is presented as percent of that in the control leaves. Error bars represent the standard deviation of the mean of seven replicates.

Each experiment was repeated at least 3 times and yielded similar results each time.

Cold activation of BN115 requires reorganization of cytoskeleton

Cold acclimatization has been shown to require rearrangements of cytoskeleton components, microtubules and actin microfilaments (Örvar *et al.*, 2000). The chemicals that cause the rearrangements of these cytoskeletal structures also cause elevation in cytosolic Ca²⁺ levels (Mazars *et al.*, 1997; Thion *et al.*, 1996). We therefore investigated the role of the stability of microfilaments and microtubules in low temperature sensing leading to the activation of *BN115*. First, the effects of microfilament stabilizer jasplakinolide (JK) and the microtubule stabilizer taxol (Tax) were determined. Both JK and Tax inhibited the cold-induced (Figure 4a), but not the constitutive (Figure 4b) GUS activity. Each of these chemicals also inhibited the cold-induced accumulation of *BN115* transcripts (Figure 4c) and caused increased solute leakage from the leaves (Figure 4d). Therefore, it may be concluded that stabilizers of microfilaments and microtubules prevent cold-induced *BN115* activation and the development of freezing tolerance.

Next, the effects of microfilament destabilizer latrunculin B (LatB) and the microtubule destabilizers oryzalin (Ory) and colchicine (Col) were examined. We reasoned that if stabilizers of cytoskeleton prevent cold-induced activation of *BN115* and development of freezing tolerance, then destabilization of the cytoskeleton may mimic the effects of cold at 25°C. Each of the three destabilizers used induced the *BN115*-driven GUS expression at 25°C (Figure 4e) but had no effect on the constitutively expressed GUS activity (Figure 4f). The accumulation of *BN115* transcripts was also induced by each of the destabilizers at 25°C (Figure 4g). However, the cytoskeleton destabilizers had little effect on solute leakage (Figure 4h). Therefore, it may be concluded that destabilizers of microfilaments and microtubules cause the activation of *BN115* but do not enhance freezing tolerance at 25°C.

Cold activation of BN115 involves action of several protein kinases and inhibition of protein phosphatases type 1 and 2A

Cold-induced gene expression and cold acclimatization have been shown to require rapid and reversible phosphorylation of specific pre-existing proteins (Monroy *et al.*, 1993b). Therefore, we wished to determine if the cold activation of *BN115* is affected by inhibitors of protein kinases and protein phosphatases. Results presented in Figure 5(a) show that general inhibitors of protein kinases, staurosporine and K252a, as well as the phosphoinositide kinase inhibitor wortmannin and the tyrosine kinase inhibitor genistein were each able to drastically reduce the cold-induction of GUS activity and *BN115* transcript accumulation. Although not much is known about protein

kinase C in plants, its inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride (H7) caused a strong reduction in the cold activation of *BN115* promoter as measured by either GUS activity (Figure 5a) or *BN115* transcript accumulation (Figure 5c). The inhibitor of cAMP-dependent protein kinases, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H8) had little effect on the cold-induced activity of *BN115*. None of the protein kinase inhibitors had any effects on the constitutively expressed GUS activity (Figure 5b). Those protein kinase inhibitors that reduced the cold-activation of GUS activity and *BN115* transcript accumulation, also reduced the development of freezing tolerance as shown by the increased solute leakage (Figure 5d). These observations suggest that activities of several types of protein kinases are required for the cold-induced activation of *BN115* and development of freezing tolerance.

Next, the effects of inhibitors of protein phosphatases were examined. Since 85% of cellular protein phosphatase activity is attributed to protein phosphatases 1 and 2A, this study focused on the effects of inhibitors of these phosphatases. Okadaic acid (OA) or calyculin A (CalyA), each a potent inhibitor of PP1 and PP2A at low concentrations, had no effect on constitutively expressed GUS activity (Figure 5f) but was able to induce *BN115*-driven GUS activity (Figure 5e) as well as the accumulation of *BN115* transcripts (Figure 5g) at 25°C. These protein phosphatase inhibitors also caused an increase in freezing tolerance as shown by the decrease in solute leakage (Figure 5h). It was therefore concluded that cold-induced activation of *BN115* promoter and development of freezing tolerance may be associated with inactivation of PP1, PP2A or both.

Discussion

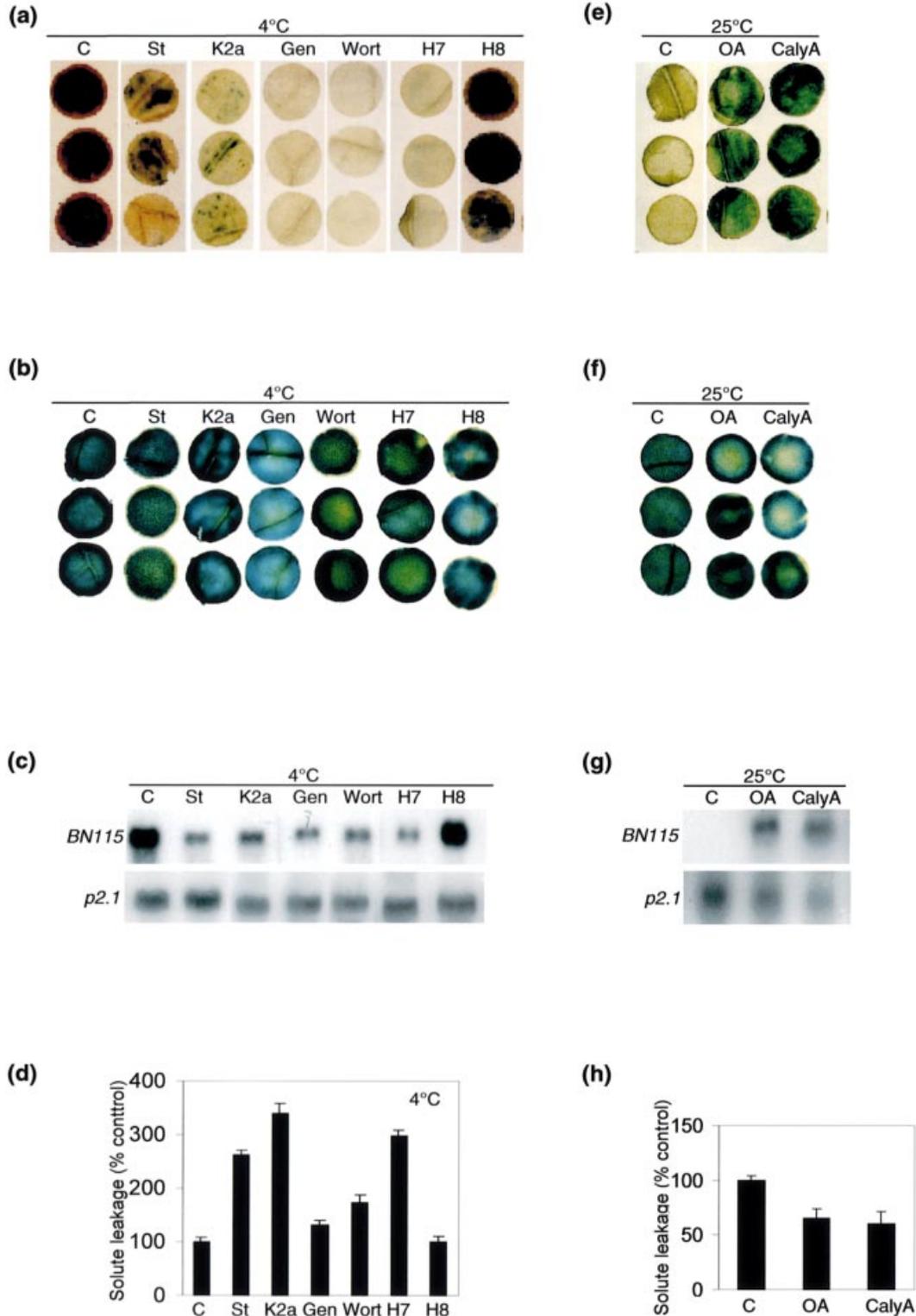
We examined the nature of early events during cold signaling in *B. napus* seedlings by monitoring (1) the activity of *BN115* promoter at the transcriptional and translational levels; and (2) the development of freezing tolerance in *Brassica napus* seedlings.

The direct and rapid effects of temperature on the physical state of biological membranes have been known for a long time (Levitt, 1980), and temperature-induced change in membrane fluidity has been considered a primary temperature sensing mechanism (Horvath *et al.*, 1998; Murata and Los, 1997; Örvar *et al.* 2000). Thus, membrane rigidification by catalytic hydrogenation in *Synechocystis* (Vigh *et al.*, 1993) and by DMSO in alfalfa cells (Örvar *et al.*, 2000), results in the expression of cold-inducible genes at 25°C. The results of the present study show that cold activation of *BN115* in *Brassica napus* leaves is prevented by membrane fluidizer benzyl alcohol and is mimicked at 25°C by the membrane rigidifier DMSO.

The effects of cold, BA or DMSO are specific to the cold-inducible *BN115* promoter as these treatments have little effect on the constitutive expression of GUS under the control of the *tCUP* promoter. It is noteworthy that DMSO

treatment mimics the effects of cold completely including the development of freezing tolerance.

There is evidence in the literature that cold-induced gene expression and cold acclimatization in alfalfa (Monroy and



Dhindsa, 1995; Monroy *et al.*, 1993b) and *Arabidopsis* (Tahtiharju *et al.*, 1997) requires Ca^{2+} influx into the cytosol. A temperature of 2°–5°C is routinely used in the laboratory to carry out cold acclimatization of plant cells and seedlings. Similar temperatures are known to activate mechanosensitive or stretch-activated Ca^{2+} channels (Ding and Pickard, 1993) and to cause maximum Ca^{2+} influx (Monroy and Dhindsa, 1995). The source of cold-induced Ca^{2+} influx has been shown to be largely cell wall in alfalfa (Monroy and Dhindsa, 1995) and *Arabidopsis* (Knight *et al.*, 1996; Tahtiharju *et al.*, 1997). An intriguing observation in the present study is that none of the two main sources of Ca^{2+} in plant cells, cell wall and the intracellular stores, by itself appears to be sufficient in causing the maximum activation of *BN115*. For example Gd^{3+} , a specific blocker of mechanosensitive Ca^{2+} channels, caused a complete inhibition of cold-induced GUS activity as well as *BN115* transcript accumulation (Figure 2a,b) while Ca^{2+} influx from intracellular stores was not blocked. This would suggest that the role of intracellular Ca^{2+} is insignificant. However, this notion is countered by the observed effects of RuR (Figure 2e,f), believed to block the release of Ca^{2+} from internal stores only (Allen *et al.*, 1995). RuR completely abolished the cold-induced GUS activity and drastically reduced the accumulation of *BN115* transcripts while Ca^{2+} entry from the cell wall was not blocked. Thus it appears that Ca^{2+} influx from both the cell wall and intracellular stores is required for low temperature signal transduction leading to *BN115* activation. The combined contribution of these two sources of cold-triggered Ca^{2+} influx has not been observed before. However, it should be noted that previous studies either did not include gene expression at all (Knight *et al.*, 1991), or monitored only the transcriptional level of gene expression (Knight *et al.*, 1996; Monroy and Dhindsa, 1995; Tahtiharju *et al.*, 1997). The present study examined the effects of Ca^{2+} availability on gene expression at the transcriptional and translational levels.

We have previously suggested (Monroy and Dhindsa, 1995) that the cold-induced membrane rigidification may be coupled to the opening of mechanosensitive Ca^{2+} channels. However, the nature of the coupling processes is unclear. Using alfalfa cells, we have recently provided

evidence that cytoskeleton rearrangements may mediate the transduction of the cold signal from the rigidified membrane to the Ca^{2+} channels (Örvar *et al.*, 2000). Remodeling of cytoskeleton is known to mediate cell responses to a variety of signals (Mathur *et al.*, 1999) and it has been suggested that cytoskeleton acts as a scaffold to the changes which transduce physical forces into biochemical signals (Schmidt and Hall, 1998). This suggestion assumes particular significance because cold activated Ca^{2+} channels have been shown to be mechanosensitive in nature (Ding and Pickard, 1993) and because Gd^{3+} , a blocker of mechanosensitive Ca^{2+} channels, prevents the cold activation of *BN115* almost completely (Figure 2a,c). Changes in membrane fluidity are likely to alter tensile forces operating in the membrane. It is well established that cytoskeleton components are attached to the plasma membrane and ion channels (Trewavas and Malho, 1998). If cytoskeleton remodeling is required for cold signaling, then the stabilizers of microfilaments and microtubules are expected to prevent it whereas their destabilizers/depolymerizers should initiate it. The results of the present study show that treatment of leaves with either the microtubule stabilizer taxol or the microfilament stabilizer jasplakinolide strongly inhibits the cold activation of *BN115* promoter. On the other hand, the microtubule destabilizer oryzalin or colchicine, or the microfilament destabilizer latrunculin B, activates the *BN115* promoter at 25°C. This suggests that cold activation of *BN115* requires reorganization of both microtubules and microfilaments. Destabilization of microfilaments and microtubules causes Ca^{2+} influx in plant cells (Mazars *et al.*, 1997; Thion *et al.*, 1996), whereas a stabilization of the actin microfilaments inhibits the cold-induced Ca^{2+} influx, gene expression and development of freezing tolerance in alfalfa cells (Örvar *et al.*, 2000).

Cold- or chemically-induced *BN115*-driven expression of GUS activity demonstrates the regulation of *BN115* at the translational level. The effects of chemicals that prevent cold activation of *BN115* (such as membrane fluidizers, cytoskeleton stabilizers and Ca^{2+} chelators and channel blockers) are consistently more pronounced on the GUS activity than on the level of *BN115* transcripts. This is especially true of the effects of Ruthenium red (RuR, Figure

Figure 5. Role of protein kinases and phosphatases in the cold-activation of *BN115*.

(a–d) Leaves were treated with general inhibitors of protein kinases staurosporine (St, 3.33 μM) or K252a (K2a, 0.67 μM), tyrosine kinase inhibitor genistein (Gen, 66.7 μM), lipid kinase inhibitor wortmannin (Wort, 8.33 μM), protein kinase C inhibitor H7 (100 μM) or cAMP-dependent protein kinase inhibitor H8 (100 μM) first at 25°C for 3 h and then continued at 4°C for 48 h. Effects of these protein kinase inhibitors on cold-induced GUS activity (a), constitutively expressed GUS activity (b), accumulation of *BN115* transcripts (c, upper panel), constitutively expressed *p2.1* transcripts (c, lower panel), and on development of freezing tolerance (d). Solute leakage is expressed as percent of that in untreated 6-day cold acclimated leaves.

(e–h) Leaves were treated with inhibitors of protein phosphatases type 1 and 2A, okadaic acid (OA, 1 nM) or calyculin A (CalyA, 1 nM) for 12 h at 25°C. Effects of OA or CalyA on *BN115*-driven GUS activity (e), constitutively expressed GUS activity (f), accumulation of *BN115* transcripts (g), and freezing tolerance (h). Solute leakage is presented as percent of that in untreated control leaves. Error bars represent the standard deviation of the mean of seven replicates. Each experiment was repeated at least 3 times and yielded similar results each time.

2a,c). Our data show that the observed effects of RuR are specifically on the cold-inducible *BN115* activity because RuR has no effect on the constitutively expressed GUS activity under the control of *tCUP* promoter (Figure 1b) and, at the concentrations used, RuR had no effect on protein synthesis (Figure 3).

The effects of several chemicals that mimic the effects of cold and activate *BN115* at 25°C are more pronounced on GUS activity than on the level of *BN115* transcripts, although both genes are transcribed under control of the same promoter. This may reflect possible differences between the stability of mRNAs of the two genes. The transcripts of cold-inducible genes are known to be extremely unstable at 25°C. For example, the half life of *cas18* transcripts in alfalfa is greater than 100 h at 4°C but less than 30 min at 25°C (Wolfrain *et al.*, 1993). Since *GUS* gene under its own promoter is not cold inducible, its transcripts, even when made under the control of *BN115* promoter, may be much more stable than the endogenous *BN115* transcripts at 25°C. Thus the chemical induction of *BN115* at 25°C is seen to be much lower in terms of *BN115* transcript accumulation than in terms of GUS activity. Cold- or chemically-induced GUS activity demonstrates that the regulation of *BN115* by these treatments is manifested at the translational level. However, our data do not rule out the possibility that the same treatments may regulate the endogenous *BN115* protein levels differently.

Mediation of cold acclimatization by protein phosphorylation has been demonstrated (Dhindsa *et al.*, 1998; Monroy *et al.*, 1993b; Tahtiharju *et al.*, 1997) and evidence for the differential roles of protein kinases and protein phosphatases in cold-induced gene expression has been reported (Monroy *et al.*, 1997, 1998). In addition to the general inhibitors of protein kinases, staurosporine and K252a, the specific inhibitors of tyrosine kinases (genistein), phosphoinositide kinases (wortmannin), and of protein kinase C (H7), all strongly inhibit the cold activation of *BN115* (Figure 4). The strongest inhibition is by genistein, wortmannin and H7. These data suggest that cold signaling, as expected, is a complex multistep process and involves the role of several types of protein kinases. The inhibition of cold activation of *BN115* by the protein kinase C (PKC) inhibitor H7 is interesting because the structural and catalytic features and role of this enzyme in plants are still unclear.

It should be noted that all chemical treatments that mimic the effects of cold in activating the *BN115* promoter at 25°C, except the destabilizers of cytoskeleton, also confer freezing tolerance. Thus they mimic all the cold-triggered events leading up to and including the development of freezing tolerance. The destabilizers of microfilaments (LatB) and microtubules (oryzalin and colchicine) do not cause any increase in freezing tolerance (or reduce

solute leakage, Figure 4h), although they activate the *BN115* promoter. While the reasons for this are presently unclear, it is likely that the continued presence of these chemicals prevents the repolymerization of the cytoskeleton in an altered pattern essential for cold acclimatization. Another microfilament destabilizer cytochalasin D had similar effects in alfalfa cells (Örvar *et al.*, 2000).

In conclusion, we have investigated the nature of events involved in the cold activation of *BN115* promoter in *Brassica napus* leaves. As end-point markers, we used the accumulation of *BN115* transcripts, *BN115*-driven GUS activity, and development of freezing tolerance. The results show that cold activation of *BN115* is mediated by membrane rigidification, cytoskeleton rearrangements and Ca²⁺ influx and involves the role of several types of protein kinases. Using alfalfa cell suspensions, we have recently provided evidence that cold signal is transmitted from the rigidified membrane to the Ca²⁺ channels via rearrangements in cytoskeleton (Örvar *et al.*, 2000). The challenge now is to identify the nature of cold-triggered rearrangements in cytoskeleton and mechanisms leading to the opening of Ca²⁺ channels.

Experimental procedures

Plant material and cold acclimation

Three-w-old transgenic seedlings of *Brassica napus* cv. Westar were used in all experiments. In addition to the endogenous cold-inducible *BN115* gene, the seedlings also contained the coding sequence of β -glucuronidase (GUS) reporter gene rendered cold inducible by its fusion to the *BN115* promoter (White *et al.*, 1994). Another transgenic line of *B. napus* cv. Westar expressed GUS activity constitutively. It carried the GUS coding sequence under control of the tobacco cryptic constitutive promoter *tCUP* (Foster *et al.*, 1999). The seedlings of this line were used as control constitutive GUS expression as opposed to the cold-inducible *BN115*-driven GUS expression. In all experiments, constitutive GUS expression refers to the *tCUP*-driven GUS expression. All other determinations were made on seedlings carrying *BN115*-GUS fusion. All plants were grown at 20°C under a 16-h photoperiod and 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. Cold treatment was administered by placing leaves at 4°C under low light intensity (20 $\mu\text{mol m}^{-2}\text{s}^{-1}$).

Chemical treatments

In order to administer chemical treatments, leaves of equal surface area were cut diagonally at the base of the petiole with a sharp razor and placed in half strength Hoagland's solution containing 0.02% Tween-20, with or without the treatment chemical. Thus chemicals were administered to the leaves via the transpiration stream through the petioles. Leaves to be exposed to cold were treated with the respective chemical first at 25°C for 3 h and then the treatment was continued at 4°C for times indicated in the respective figure legends. Leaves to be maintained at 25°C were treated with the chemical at 25°C for times indicated. Harvesting involved cutting the leaf blade at the

mid-rib and immediately freezing one half of the leaf in liquid nitrogen for RNA extractions. Leaf discs were punched out from the other half of the leaf and used to perform GUS assays. *BN115* is not induced by wounding (Weretilnyk *et al.*, 1993). In all experiments where a solvent other than water was used to dissolve the treatment chemical, control incubation medium (C) contained the same solvent concentration as the treatment medium.

Concentrations of different chemicals used in various experiments are given in respective figure legends.

Histochemical assay of GUS activity

At termination of the experiment, leaf discs were punched out and washed in 90% acetone for 15 min and then rinsed for 5 min in rinse solution (50 mM sodium phosphate, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$). The discs were then placed for 24–48 h in rinse solution containing 1.5 mM 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc, Rose Scientific, Edmonton, Alberta, Canada) and 0.05% Triton-X-100. Chlorophyll was removed by washing discs in 30% ethanol for 1 h, and in 50% FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 1 h. Discs were then stored in 70% ethanol until photographed.

Extraction of total RNA

Leaves were ground in liquid nitrogen and RNA was extracted using TRIzol (Gibco BRL, Burlington, Ontario, Canada), following the manufacturer's instructions.

Preparation of cDNA probe

A plasmid containing *BN115* cDNA, *pBN115*, was cut with EcoRI and the released 800-bp fragment, corresponding to the *BN115* cDNA, was gel-purified and radiolabeled by random priming using the T7 QuickPrime® Kit (Pharmacia; Amersham Pharmacia, Baie d'Urfé, Québec, Canada), and [α - ^{32}P]dCTP, following the manufacturer's instructions. The constitutively expressed *p2.1* was used as a control gene as previously described (Monroy *et al.*, 1993a).

RNA gel-blot analysis

Total RNA was separated on a 1.5% formaldehyde gel, transferred to a nylon membrane (Biotrans, ICN, Costa Mesa, California, USA) overnight, UV cross-linked, and the membrane was dried. Blots were hybridized overnight (7% SDS, 0.25 M phosphate buffer, pH 7.4, 2 mM EDTA) with the probe in a Robbins scientific hybridization incubator, Model 400, at 60°C and 15 r.p.m. Washes, 15 min each at 60°C, were twice in 1X SSC, 0.1% SDS, and once in 0.1X SSC, 0.1% SDS.

Determination of solute leakage

Solute leakage was used as an inverse measure of freezing tolerance. Treated leaves were rolled and placed in Falcon 2059 tubes in a freezing water bath kept at 0°C for 30 min. Samples were then seeded with a small piece of ice and kept at 0°C for another 60 min, after which the temperature was decreased at a rate of 2°C/h to -4°C, followed by -5°C for 30 min. Samples were then placed at 4°C overnight. Water was added to the tubes and

electrolyte leakage measured after 4 h of gentle shaking at 25°C (Reading 1). Samples were placed at -80°C overnight, and the electrolyte leakage measured after shaking at 25°C for 4 h (Reading 2). Percent solute leakage was calculated by dividing reading 1 by reading 2 and multiplying the result by 100. Cold acclimatization for determining solute leakage experiments was carried out for 6 d at 4°C.

Determination of the rate of protein synthesis

Leaf discs were treated with varying concentrations of ruthenium red in half-strength Hoagland solution for 3 h at 25°C, leaf discs were punched out placed in half-strength Hoagland solution containing 50 μ Ci [^{35}S]methionine (1000 Ci $mmol^{-1}$, Amersham Pharmacia) for 3 h. The leaf discs were then washed with half strength Hoagland's solution containing 1 mg l^{-1} methionine, ground to a powder in liquid nitrogen and protein extract prepared by adding 2 volumes (w/v) of buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4 , 10 mM NaF, 50 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μ g ml^{-1} antipain, 5 μ g ml^{-1} aprotinin, 5 μ g ml^{-1} leupeptin, 10% glycerol, 7.5% polyvinylpyrrolidone). The homogenate was centrifuged for 20 min at 13 000 r.p.m. Supernatant was transferred to another tube, and protein amount was assayed using the Bradford assay (BioRad). An identical aliquot of the extract was used to precipitate proteins with 5% TCA. The radioactivity in the aqueous suspension of the proteins was determined by scintillation counting. The results presented are as means of 3 replicates with error bars representing standard deviation.

All experiments were repeated at least 3 times and yielded similar results each time.

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