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Resposes of Two Cold-Regulated Genes, BN28 and BN115, in Field-Grown Canola (*Brassica napus* L.)

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圃場에서 케놀라 低溫反應性 유전자 발현

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ABSTRACT : *Cold acclimation involves changes in gene expression. BN28 and BN115 are two* genes which are regulated by cold temperature and assumed having roles in cold acclimation. The objectives of this experiment was to explore the expression of BN28 and BN115 under field conditions. Six winter cultivars were planted at three different dates during the fall. The expression of the genes was determined by northern blot analysis of total RNA taken from leaves 15 to 30 day-intervals after planting.

The expression of the two genes was detected within 15 days after planting well before onset of freezing tolerance in plants. This suggestes either their expression was a prerequisite of the freezing tolerance or their expression was regulated by other environmental factors as well as temperature.

Two genes showed a different expression pattern suggesting they had a different regulatory system. Although time-course increase in expression of the cold-regulated genes was matched with increase in freezing tolerance, the difference of expression in cultivar level at specific times of measurement was not correlated with freezing tolerance at the moment.

Key words : Freezing tolerance, Cold-regulated gene, BN28, BN115, Canola

Although the physiological and biochemical changes that occur during cold acclimation could be brought about by preexisting structural and enzymatic changes, it is also possible, as first proposed by Weiser[24], that cold acclimation involves changes in gene expression. Direct evidence was obtained recently to show that low temperature induced accumulation of specific mRNAs. The appearance of novel transcripts during cold acclimation has been observed in alfalfa[19], wheat[13,14], barley[5,9], *Arabidopsis*[10,12,16,21], and rice[2].

In *B. napus*, two cold-regulated cDNA clones were isolated and characterized[22,25]. BN28 is homologous to *cor*6. 6 of *Arabidopsis*[10] and encodes abundant mRNA of 0.45 kilobases,

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induced by the exposure of the whole plant to 2℃, with the transcript appearing within 6 hours of exposure[22]. The transcript disappears within 20 hours of the return of plant to room temperature. ABA also induced the expression of this gene but heat shock did not. The functions of the products of BN28, *cor*6. 6 and *kin*1 are not yet known. However, based on regions of deduced amino acid homology to flounder antifreeze protein, these genes may possess antinucleation properties[9].

BN115 is another cold-regulated gene isolated from *B. napus*[24] which has sequence homology with *cor*15 of *Arabidopsis*[12]. This gene encodes abundant mRNA of 0.8 kilobases induced by cold temperature, with the transcript appearing within 1 day of exposure. The transcript disappears within 20 hours of the return of the plant to room temperature.

In contrast to BN28, ABA did not induce the expression of this gene, but dehydration leads to a slight increase in transcript at room temperature. Northern blot analysis showed that levels of transcript increased within 24 hours of exposure to low temperature, peaked after 3 days, and remain at elevated level for the duration of the cold treatment. BN115 reveals a single open reading frame that potentially encodes a protein of 14.8 kDa which is matched to that of COR15 (14.7 kDa) of *Arabidopsis*[12]. A search of GenBank and EMBL database did not reveal any proteins with significant similarity to COR15.

Although many studies have demonstrated the expression of stress-induced genes under laboratory conditions[1,3,7,12,16,17,20], few investigators have examined the expression of such genes under field conditions. Kimpel and Key[15] showed that mRNA encoding heat shock proteins (HSP) accumulated in leaf tissues of soybean experiencing high temperature in the field. Similar studies were conducted with other crop species by Burke et al.[4]. Both field experiments and growth chamber studies using conditions designed to simulate a day of high-temperature stress showed evidence that HSPs are expressed under natural conditions[6,8]. Lorraine et al.[18] examined the expression of HSPs at the whole plant level in alfalfa under field conditions. The fact that some of the HSPs are expressed in the seed and flowering parts in the absence of heat shock suggests a more widespread occurrence of HSPs in optimal growth environments and their potential role in plant development.

Expression of cold-regulated genes like *cor* genes from *Arabidopsis* and BN genes from *Brassica napus* have not been examined under field conditions.

The objectives of this research were to study the response of two cold-regulated genes, BN28 and BN115, isolated from *B. napus* under field conditions at different planting dates. In addition, the correlation between the degree of the gene expression and the increase of the freezing tolerance was also discussed.

MATERIALS AND METHODS

Six winter canola cultivars (WRG86, CDH3, Duobul, Ceres, Accord and KWC4113) were planted at three different planting dates, Aug. 25, Sept. 10, and Sept. 25 in 1993 in the field. Samplings for RNA extracton were done 15 (Sept. 10), 30 (Sept. 25), 45 (Oct. 10), and 75 (Nov. 13) days after planting (DAP) for the Aug. 25 planting; for the Sept. 10 planting, at 15 (Sept. 25), 30 (Oct. 10), and 60 DAP (Nov. 13), and for the Sept. 25 planting, at 15 (Oct. 10) and 45 DAP (Nov. 14).

Leaf and stem parts of plants were cut in the field, frozen in liquid N2, then kept in a box with dry ice before storage at −80℃ in the laboratory. Total RNA was isolated by the method of Gilmour et al.[10,11]. Frozen pulverized plant materials were extracted in a buffer containing 100 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM EGTA, 1%(w/v) SDS, 10m M DTT, 6 % (w/v) p-aminosalicylic acid (sodium salt), and 1 % (w/v) tri-isopropyl naphthalene sulfonic acid with an equal volume of buffer-saturated phenol: chloroform: isoamyl alcohol (25:24:1,PCI). After centrifugation to separate the phases, the aqueous phase was extracted once more with PCI, and the nucleic acids were ethanol-precipitated twice on ice with 2M LiCl, followed by a final ethanol precipitation. The pellet was redissolved in distilled water and stored at -80℃. Plasmid DNA was isolated by alkaline lysis, followed by polyethylene-glycol (PEG) precipitation. Whole plasmid was cut with the restriction enzyme, EcoRI, and subjected to agarose gel electrophoresis. Insert DNA from the gel slice was isolated using the Gene Clean Kit. ^{32}P-labelled probes were prepared using 64 ng of template insert DNA by the random oligonucleotide primers method.

Northern blot analysis was done as previously described with some modification[12]. Fifty *u*g of total RNA dissolved in 100 *u*L RNA loading buffer. From the 100 *u*L of sample solution, only 15 *u*L (7.5 ug of total RNA) were loaded on the gel. Total RNA was fractionated on 1.2 % denaturing formaldehyde agarose gel and northern blot was prepared on a Nytran membrane (Schleicher and Schuell) using 20x SSPE as the transfer buffer. As a check for RNA integrity, efficiency of transfer, and equivalency of loading, the EtBr stained gel and membrane were carefully examined visually under UV light. The RNA in the membrane was immobilized by cross-linking with UV liht. Prehybridization and hybridization were done according to manufacturer's instructions(Schleicher and Schuell). The transcript amount was quantified using a Betagen 603 Blot Analyzer(Betagen Corp.). Filters were stripped for subsequent rehybridization by submerging in boiling 5 mM Tris-HCl(pH 7.8), 1mM EDTA, and 0.05 % SDS for 15 min. Stripped filters were rehybridized with the radish rDNA, pRE12, and quantified as mentioned before for the standardization of total RNA present in the membrane.

The northern blot experiments were conducted three times as replications using three different RNA samples. The freezing tests were done at the same times of the RNA extraction as preuiously described[27].

RESULTS AND DISCUSSIONS

To determine the response of two-cold regulated genes under field conditions, total RNA from six cultivars planted Aug. 25 were extracted from 15, 30, 45, and 75-day old plants. Northern blot analysis was performed with BN28 and BN115 probes(Fig. 1). The general pattern of expression of the two genes is shown in Fig. 2. Each data point is the overall mean for six cultivars. Both genes were turned on within 15 days of planting; however, the expression patterns of the two genes were different. The transcript amount of BN28 increased gradually throughout the experimental period, while that of BN115 increased rapidly within 15 DAP followed by a steady increase until the end of the experiment (Nov. 13). At this point, both genes

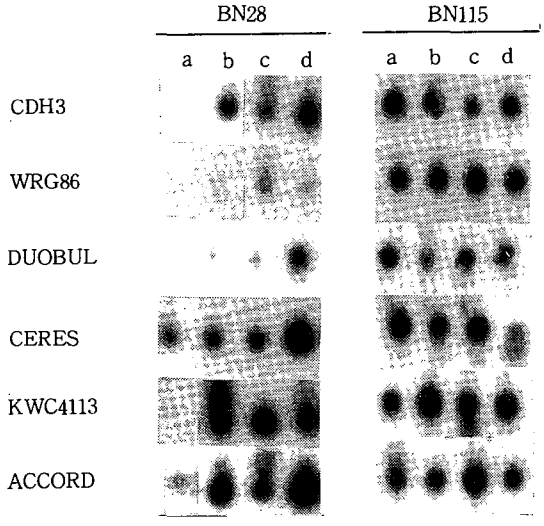

Fig. 1. Northern blot analysis of six winter canola cultivars planted Aug. 25, 1993 in the field. Total RNA were isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d). The membrane was hybridized with BN28 and BN115

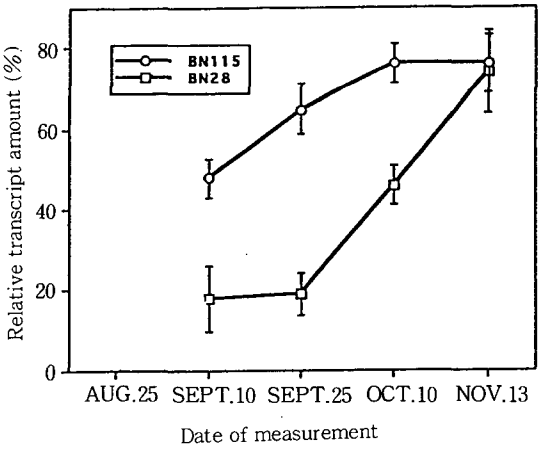

Fig. 2. Time-course changes in transcript amount of BN28 and BN115 in canola planted Aug. 25, 1993. The data represent the overall mean of six cultivars and is a graphical representation of Fig. 1. Vertical bar indicates mean± SE.

retained their full transcript level. One interesting observation is that both the genes

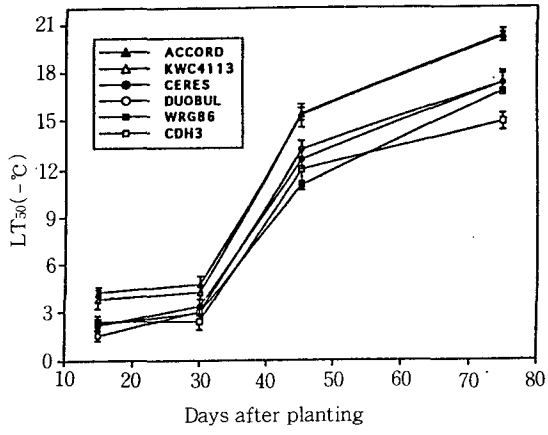

Fig. 3. Changes in freezing tolerance of six winter canola cultivars planted Aug. 25, 1993. Freezing tests were conducted at 15, 30, 45, and 75 days after planting. Bar indicates mean±SE.

Table 1. Simple correlation coefficients† between transcript amount of BN28 and BN115 and freezing tolerance of canola cultivars planted Aug. 25. Sept. 10, and Sept. 25, 1993

Planting date	r	
	BN 28	BN 115
Aug. 25	0.59ns‡	−0.37ns
Sept. 10	0.47ns	−0.33ns
Sept. 25	0.60ns	−0.40ns

† Correlation coefficient was estimated between transcript amount at Nov. 13 and freezing tolerance at Nov. 13

‡ (*) and (**) simple correlation coefficient, significant at 0.05 and 0.01 probability levels, respectively. (ns) significant at the 0.05 probability level

were turned on well before the onset of increase in freezing tolerance(Fig 3). The expression of these genes in each cultivar was examined to determine the correlation between the freezing tolerance of a specific cultivar and transcript level (Table 1).

There were no specific patterns of the gene expression to explain the difference in freez-

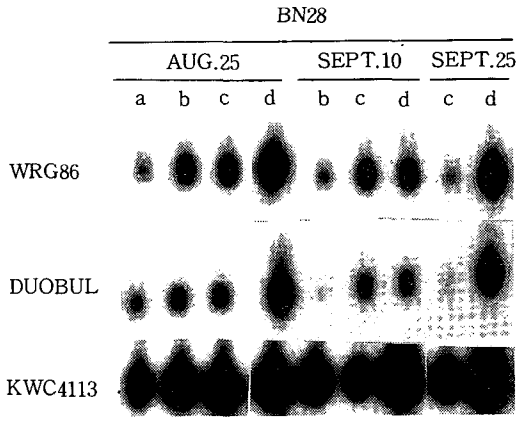

Fig. 4. Northern blot analysis of 3 winter canola cultivars planted Aug. 25, Sept. 10, and Sept. 25, 1993, respectively. Total RNA from leaves of plants was isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d), respectively. The membrane was hybridized with BN28.

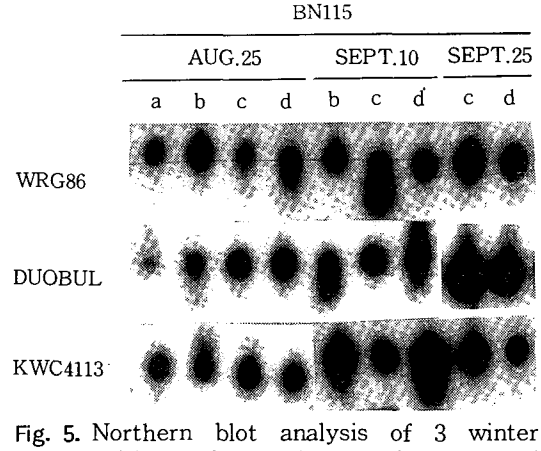

Fig. 5. Northern blot analysis of 3 winter cultivars planted Aug. 25, Sept. 10, and Sept. 25, 1993, respectively. Total RNA from leaves of plants was isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d), respectively. The membrane was hybridized with BN115.

ing tolerance among cultivars. Though most cultivars showed increases in BN28 expression over time, the differences among cultivars on a specific sampling date were inconsistant. In

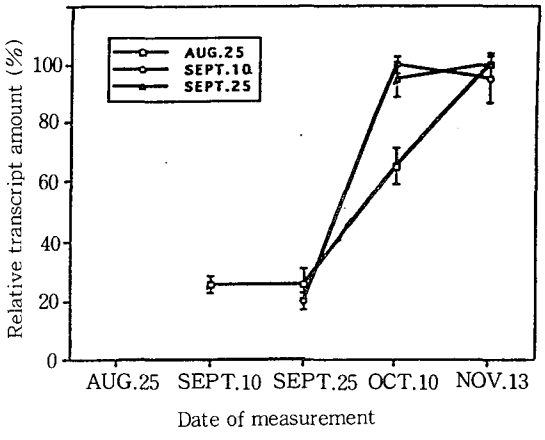

Fig. 6. Changes in transcript amount of BN28 affected by different planting dates. Data represent overall means of theree cultivars and a graphical presentation of the northern blot in Fig. 4. Vertical bar indicates mean±SE.

the case of BN115, there was no difference in gene expression at either among sampling dates or cultivars on a specific sampling date.

The effect of planting date on the expression of BN28 and BN115 was examined by northern blot analysis of total RNA from 3 winter cultivars at 3 different planting dates (Aug. 25, Sept. 10, and Sept. 25). Total RNA was probed with BN28 (Fig. 4) and BN115 (Fig. 5). The graphical representation of the gene expression affected by different planting dates is shown in Fig. 6 (BN28) and Fig. 7 (BN115). There was both a slow and rapid phase of increase in expression of BN28. In the Aug. 25 planting, the increase in expression of BN28 was slow until 30 DAP, then increased rapidly from 30 to 45 DAP. Both in the Sept. 10 and Sept. 25 plantings, the rapid increase phase was coincident with that of the Aug. 25 planting regardless of plant ages. The maximum expression was attained at the end of the experiment (Nov. 13) and there was no difference in transcript amount of BN28 at the end of experiment among the three planting

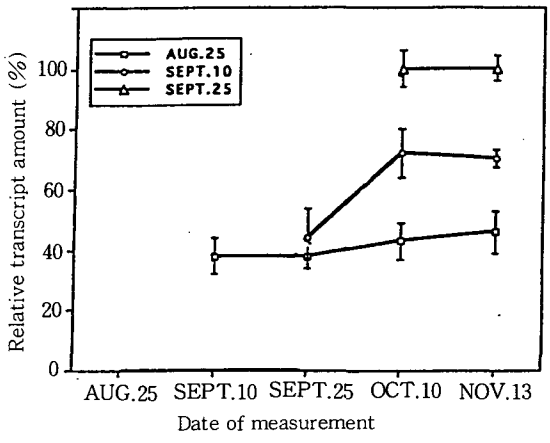

Fig. 7. Changes in transcript amount of BN115 affected by different planting dates. Data represent overall means of three cultivars and a graphical presentation of the northern blot in Fig. 5. Vertical bar indicates mean±SE.

dates. BN115 reached its full expression within 15 DAP in two planting dates and later planting induced a higher expression than earlier planting at the end of the experiment (Nov. 13).

The expression pattern of BN28 and BN115 under field conditions was similar to that in laboratory[27]. The maximum expression was attained at the begining of cold acclimation in the case of BN28, whereas expression of BN115 was increased steadily as long as the plants were remained at cold temperature.

This suggests that these two genes are regulated by different regulatory systems. The expression of the two cold-regulated genes, BN28 and BN115, revealed no clue about their roles in cold acclimation.

Though there is no correlation between freezing tolerance and expression of these genes at the cultivar level at specific sampling times, observations that these genes are turned on in response to cold temperature in the field and that the time-course increase in BN28 expression is coincident with increase in freezing tolerance suggests that they may have a role in winter hardiness. The possibility of the two cold-regulated genes as molecular markers for selection for winter hardiness is not promising.

The expression of these genes under field conditions well before the onset of increase in freezing tolerance[27] suggestes two possibilities for the function and regulation of the genes. The expression of the genes is a prerequisite of gaining of freezing tolerance simply because these genes were turned on before onset of freezing tolerance and time-sequence expression of the two genes was well matched with that of the freezing tolerance. Another possibility is that other environmental factors than temperature is a regulatory signals for the expression of the genes under field conditions. The fact that the genes were turned on by the treatment of ABA or drought under laboratory condition supports this assertion if we assume that the unknown environmental factors, for example, photoperiod changes the internal conditions of plants having high endogeneous ABA concentration, which leads the expression of the genes.

In summary, the results indicated that expression of the genes were occured under field conditions and the results of this field studies not only suppots the idea that the genes are regulated by temperature under field condition but also the idea that the genes may be regulated by other environmenmtal factors which influence the internal conditions of plants. How these genes are regulated under field condition are remained to be determined.

摘　　要

圃場狀態에서의 케놀라의 低溫反應性 遺傳子인 BN28 과 BN115 의 發現程度와 그 유전자들의 耐凍性에 대한 역할을 구명하기 위하여 6개 품종 (WRG86, CDH3, Dusul Ceres, Accord, KWC-4113)을 파종기를 달리하여 파종하고 파종후 15일 간격으로 Sample를 채취하여 RNA를 추출하고 northern blot analysis로써 두 遺傳子의 發現정도를 조사하였던바 그 결과를 요약하면 아래와 같다.

1. 두 遺傳子의 發現시기는 耐凍性이 증가하는 시기보다 조금 앞서는 것으로 나타났으며 이는 식물체가 耐凍性을 얻기 위하여 이 유전자의 발현을 필요로 하거나 혹은 이 遺傳子 發現을 調節하는 要因중 온도 이외의 環境要因이 관여하는 것으로 나타났다. 하지만 유전자 발현이 급격히 증가하는 시기가 세 파종시기 처리에 있어서 일치하는 점과 또한 耐凍性의 급격한 증가시기와 일치하는 점으로 미루어 보아 이 遺傳子 發現을 調節하는 가장 중요한 요인은 低溫인 것으로 생각된다.
2. 發現樣式에 있어서 두 遺傳子간의 차이가 관찰되었는데 이는 두 유전자가 각각 다른 調節機作을 통하여 발현이 통제되는것으로 생각되며 이러한 포장에서의 發現樣式은 실험실에서의 그것과 일치하는 것으로 나타났다.
3. 遺傳子가 발현되어 증가되는 시기는 식물체의 耐凍性이 증가되는 시기와 일치되었다. 하지만 특정한 시점에 있어서 각 품종간의 耐凍性의 程度와 遺傳子 發現程度는 일치하지 아니하였다.

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