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Salicylic Acid and Wounding Induce Defense-Related Proteins in Chinese Cabbage

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The response of plants to pathogens and wounding is dependent upon very sensitive perception mechanisms. Although genetic approaches have revealed a variety of resistance genes that activate common defense responses, defense-related proteins are not well characterized in plants. Therefore, we used a proteomic approach to determine which defense-related proteins are induced by salicylic acid (SA) and wounding in Chinese cabbage. We found that SA and wounding induce pathogenesis-related protein 1a (PR1a) at both protein and mRNA levels using proteomics and Northern blot analysis, respectively. This indicates that our proteomic approach is useful for identifying defense-related proteins. We also identified several other proteins that are induced by SA or wounding. Among the seven SA-induced proteins identified, four may be defense-related, including defense-related protein, phospholipase D (PLD), resistance protein RPS2 homolog, and L-ascorbate peroxidase. Out of the six wounding-induced proteins identified, three may be defense-related: heat shock cognate protein 70 (HSC70), polygalacturonase, and peroxidase P7. The precise functions of these proteins in plant defense responses await further study. However, identification of the defense-related proteins described in this study should allow us to better understand the mechanisms and signal transduction pathways involved in defense responses in Chinese cabbage.

Plants quickly respond to a pathogenic attack by activating inducible defense responses (Hutcheson, 1998; Dangl and Jones, 2001; Lam et al., 2001). Activation of such responses occurs upon pathogen recognition and triggers programmed cell death, called the hypersensitive response (HR), which results in rapid execution of defense responses such as systemic acquired resistance (SAR) (Hunt et al., 1996; Ryals et al., 1996; Heath, 2000). Salicylic acid (SA) plays a central role in the signaling pathways involved in SAR (Vernooij, 1994; Dong, 1998). SA is derived from phenylalanine during the defense response, but it is not known how it is regulated since the regulatory mechanisms may differ among plant species and a wide variety of genes are induced (Hunt et al., 1996). For example, SA inhibits catalase activity, which leads to elevated H_2O_2 levels, induces pathogenesis-related (PR) gene expression, and induces SAR in tobacco (Chen at al., 1993). However, several reports have established that H_2O_2 is not a second messenger of SA during SAR (Ryals et al., 1996). Although the mechanism by which SA induces SAR is unclear, systemic accumulation of SA has been shown to strongly induce expression of many defense-related genes, including *PR1* (Epple et al., 1995), *BGL2* (Uknes et al., 1992), *HEL* (Potter et al., 1993), *PR-5* (Uknes et al., 1992; Epple et al., 1995), and *TLP1* (Hu and Reddy, 1997), in a variety of plant species (Reymond and Farmer, 1998).

In nature, mechanical wounding occurs when insects feed on plant tissues. Plants respond to wounding by inducing a defense response that is characterized by the expression of a certain set of genes (Hildmann et al., 1992). This response involves reinforcement of the cell wall by accumulation of lignin (Bernards and Razem, 2001), extensins (Jose-Estanyol and Puigdomenech, 2000), proline-rich protein (Vignols et al., 1999), and hydroxy-proline-rich glycoproteins (Corbin et al., 1987) as well as production of protease inhibitors such as systemin (Bowles, 1990; Bergey et al., 1996). Jasmonic acid (JA) is one of the most powerful inducers of proteinase inhibitors in plants (Farmer and Ryan, 1992). As a key molecule involved in wound response, JA induces the expression of a number of pathogenesis-related genes, including *THI2.1* (Vignutelli et al., 1998), *PDF1.2*

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(Penninckx et al., 1996), *CHIA1* (Samac and Shah, 1994), *HEL* (Potter et al., 1993), and *CHIB1* (Samac and Shah, 1991).

SA and JA, which are both low-molecular weight regulators, are essential for local defense reactions as well as for induction of systemic resistance (Creelman and Mullet, 1997; Durner et al., 1997). The relative contribution of each of these signaling molecules to the regulation of defense responses and to the induction of defense gene expression depends upon both the pathogens and plant species involved. Despite a wide variety of pathogens and plants, the defense-related genes and signaling pathways that are involved in these processes are relatively well understood. However, the pathogenesis-related proteins that are induced by SA and wounding are not known. In this study, we employed a proteomics technique to determine which defense-related proteins are induced by SA or wounding in Chinese cabbage. Several proteins, including pathogenesis-related protein 1a (PR1a), that are induced by SA or wounding are described.

Materials and Methods

Plant materials

Chinese cabbage (*Brassica rapa* subsp. *pekinensis* cultivar Norang) seedlings were grown in a green house (70% humidity, 25°C) for 3 months. Whole leaves were sprayed with 20 mM MOPS (pH 7.5) for control or 20 mM MOPS (pH 7.5) containing 5 mM SA for SA treatment for 24 h at room temperature. For the wounding treatment, leaves were cut into 1-cm × 1-cm squares using a razor blade and were then incubated for 24 h at room temperature while being sprayed with 20 mM MOPS (pH 7.5). Leaves were harvested, weighed, and quickly frozen in liquid nitrogen and then stored at -80°C until use.

Preparation of total protein extracts

Total proteins were homogenized with extraction buffer (80 mM Tris buffer, pH 7.5, 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). The extract was then squeezed through four layers of cheese-cloth. Nucleic acids and pectins were precipitated by adding 0.1% (v/v) polyethyleneimine (pH 7.5). After stirring for 20 min at 4°C, the extract was centrifuged for 30 min at 13,000×g and the pellet was discarded. Ammonium sulfate (100%) was then added to the supernatant, which was stirred for 30 min at 4°C and centrifuged for 30 min at 20,000×g. The resultant pellet was resuspended in extraction buffer and dialyzed against the same buffer for 24 h at 4°C. The protein solution was clarified by centrifugation for 10 min at 48,000×g and dried by a vacuum freeze dryer. Dried proteins were resuspended in lysis buffer (8 M urea, 4% Triton X-100, and 40 mM Trisma base). Protein concentration was estimated according to the Bradford method with IgG as a standard protein (Bradford, 1976).

Two-dimensional electrophoresis

Proteins were first separated by electrophoresis according to charge. Isoelectricfocusing (IEF) was carried out with 1 mg proteins using gel strips that formed an immobilized linear pH gradient (IPG, pH 3–10) that was 13 cm long. The strip was rehydrated for 16 h at 25°C with rehydration solution (8 M urea, 2% Triton X-100, 2% IPG buffer, 20 mM DTT, and bromophenol blue). IEF was performed at 22°C in the Multiphor II system (Amersham Pharmacia Biotech) for 2 min at 300 V (2 mA and 5 W) and 5.5 h at 3,500 V (2 mA and 5 W).

After IEF, the IPG strip was incubated with equilibration solution (1.5 M Tris-HCl buffer, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 65 mM DTT, and 135 mM iodoacetamide) for 15 min at room temperature. Proteins were then separated according to size. The equilibrated gel strip was placed on top of a vertical 12% (w/v) polyacrylamide gel and was sealed with 1% agarose. After agarose solidification, electrophoresis was performed in 25 mM Tris buffer (pH 8.0) containing 192 mM glycine and 0.1% (w/v) SDS for 2 h at 35 mA. The gel was stained with Coomassie brilliant blue.

Identification of protein

Protein spots of interest were excised from the Coomassie blue-stained 2-dimensional (2-D) gel. For destaining, each gel piece was washed with 25 mM ammonium bicarbonate/50% acetonitrile (pH 8.0) followed by 100% acetonitrile, and this was repeated until the gel pieces were completely destained. Complete dehydration was then performed with Speed-Vac. The dried gel pieces were added to 25 μl trypsin (10 μg/ml) solution containing 25 mM ammonium bicarbonate (pH 8.0) and incubated at 37°C for 16 h. Twenty-five μl of 50% acetonitrile/5.0% TFA were then added and the samples were incubated for 1 h; this step was repeated one more time. The supernatant was then dried by Speed-Vac. Protein fragments digested by trypsin were characterized by mass spectrometry and then peptide mass fingerprinting was obtained using a PE Biosystems Voyager System 6148. Proteins were identified by searching protein sequence databases (MS-Fit, http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) using the tryptic peptide molecular mass.

Northern blot analysis

Northern blot analysis was performed using standard procedures with a DIG High Prime DNA Labeling and

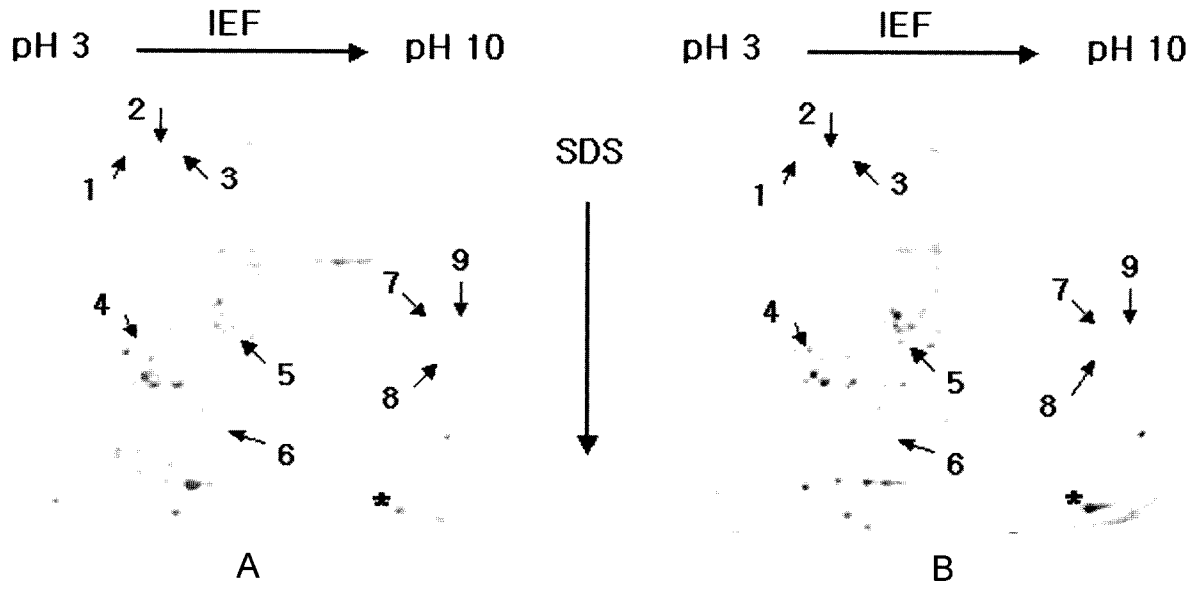

Fig. 1. Coomassie blue-stained 2-D gel separation pattern of the total proteins prepared from unstimulated (A) and SA-stimulated (B) Chinese cabbage leaves. Arrows indicate protein spots that are strongly induced following treatment with 5 mM SA for 24 h. Asterisk was identified as PR1a protein.

Detection Starter Kit II (Roche Molecular Biochemicals, Ryang et al., 2002). Briefly, 10 μg total RNA were separated on a 1.0% formaldehyde-agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) using the standard capillary transfer method. After UV cross-linking at 125 mJ, blots were hybridized using the digoxigenin (DIG)-labeled DNA probe, which was detected using an alkaline phosphatase-conjugated anti-DIG antibody with CSPD as a chemiluminescent substrate. The DNA probe was prepared by PCR amplification of the cDNA corresponding to PR1a. The oligonucleotides (5'-GGAGCTCTTGTTCATCCCTC-3' and 5'-ATTATGTGAACGAGAAGCCTTAC-3') were used as primers for preparation of the cDNA.

Results

Identification of PR1a as a protein induced by SA and wounding

Proteins were extracted from SA-treated and untreated Chinese cabbage leaves and analyzed by 2-D gel electrophoresis. A distinct protein spot was identified at a low-molecular weight and high-pI gel position from leaves treated with 5 mM SA for 24 h; the intensity of the spot was much stronger than that on the gel containing untreated samples (Asterisk in Fig. 1). We used proteomic technique to identify the protein. The protein was digested in the gel with trypsin, and a mass spectrum of the resulting peptides was acquired with a matrix-assisted laser-desorption ionization time of flight (MALDI-TOF) mass spectrometer. We then submitted the resultant 16 peaks to MS-Fit to determine the identity of the protein spot. The search selected pathogenesis-related protein 1a (PR1a) as the most likely candidate for the spot. Thirty-four percent of the PR1a sequence was spanned

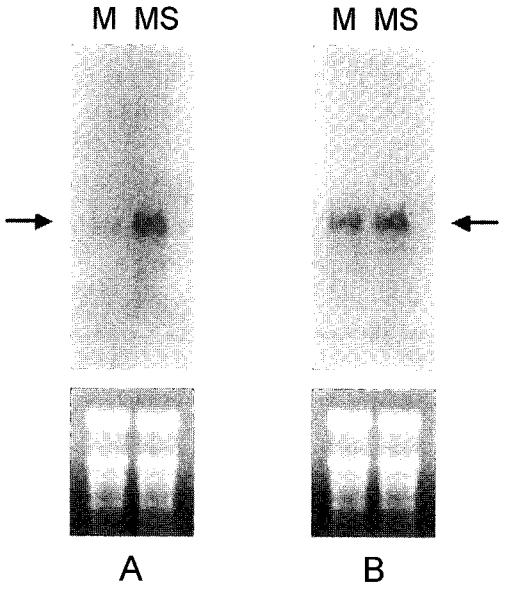

Fig. 2. Detection of PR1a mRNA by Northern blot analysis. Each lane contains 20 μg of total RNA from (A) whole leaves and (B) 1-cm×1-cm cut leaves (wounding). M and MS indicate treatment with MOPS buffer and MOPS buffer containing 5 mM SA, respectively. Arrows indicate PR1a. As a control, the same amount of total RNA separated on an agarose gel is shown at lower panel of the figure.

Table 1. Identification of proteins induced by salicylic acid using a proteomic approach

Spot Number	Protein MW(Da)/pI	Species	Putative Identification	Accession Number	Identity (%)
1	92062/5.37	*Brassica oleracea*	Phospholipase D 2 precusor	13124446	88
2	92062/5.37	*B. oleracea*	Phospholipase D 2 precusor	13124446	67
3	92062/5.37	*B. oleracea*	Phospholipase D 2 precusor	13124446	67
4	39183/5.37	*B. carinata*	Defense-related protein	14009292	78
5	36460/6.10	*B. napus*	Arginase	13182957	68
6	27638/5.70	*B. napus*	L-ascorbate peroxidase	1890354	48
7	40624/8.91	*B. rapa*	S-locus related 2-9A	16040956	67
8	33460/8.65	*B. oleracea*	Resistance protein RPS2 homolog	6503054	76
9	41387/9.05	*B. napus*	Protein serine/threonine kinase BNK1	10445209	60

by the tryptic fragments (data not shown).

To determine whether PR1a is induced by SA at the RNA level, we performed Northern blot analysis using the DIG High Prime DNA Labeling and Detection system. As shown in Fig. 2A, the steady state level of PR1a mRNA was drastically increased in SA-treated leaves. Interestingly, PR1a gene expression was also induced by wounding (Fig. 2B). The proteomic approach used to identify PR1a therefore appeared to be successful, so we used it to find additional proteins that are induced by SA or wounding.

Analysis of defense-related proteins induced by SA

Comparison of Fig. 1A and B reveals that a large number of proteins are enriched in the total protein preparation of SA-stimulated Chinese cabbage leaves. In the current study we initially focused on nine spots, designated 1 - 9 in Fig. 1. These spots were excised and digested in the gel with trypsin overnight. The resulting peptide mixtures were analyzed by MALDI-TOF MS in the reflection mode. Proteins were identified using an MS-fit algorithm that allowed a peptide mass tolerance of ±0.2 Da. Information about the identified protein spots is summarized in Table 1. Plant defense proteins such as phospholipase D (PLD), defense-related protein, and resistance protein RPS2 homolog were identified. In addition, L-ascorbate peroxidase, S-locus related 2-9A, and protein serine/threonine kinase BNK1 were also identified. However, it is not clear whether these proteins are involved in SA-regulated defense mechanisms in Chinese cabbage.

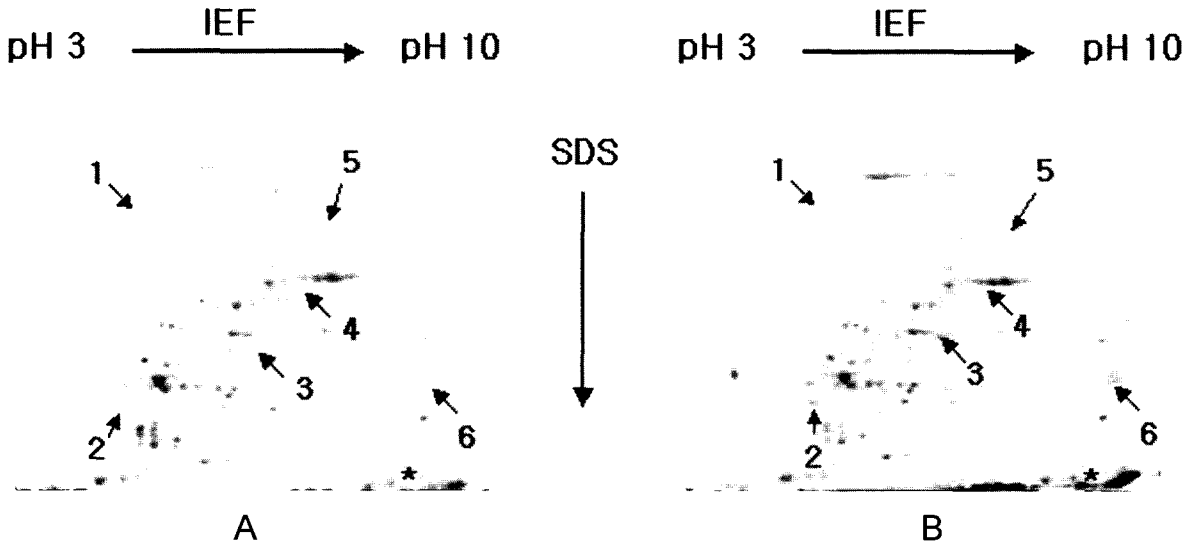

Fig. 3. Proteins induced by wounding. Coomassie blue-stained 2-D gel separation pattern of the total proteins prepared from (A) whole (control) and (B) 1-cm×1-cm cut (wounding) Chinese cabbage leaves. Arrows indicate protein spots that are largely induced following treatment of wounding. Asterisk indicates PR1a protein.

Table 2. Identification of proteins induced by wounding using a proteomic approach

Spot Number	Protein MW(Da)/pI	Species	Putative Identification	Accession Number	Identity (%)
1	70774/5.08	*Brassica napus*	Heat shock cognate protein HSC70	2655420	88
2	27745/4.97	*B. napus*	Lysine-ketoglutarate reductase/saccharopine dehydrogenase	11065906	40
3	42447/6.34	*B. napus*	Polygalacturonase precursor (PG) (Pectinase)	548490	82
4	49589/7.43	*B. rapa*	SLG12	1304009	51
5	69047/7.72	*B. juncea*	Efflux carrier, pin2	15485153	80
6	31086/9.10	*B. rapa*	Peroxidase P7	464365	73

Analysis of defense-related proteins induced by wounding

A 2-D electrophoresis map was constructed to identify defense-related proteins induced by wounding (Fig. 3). Protein spots of interest (designated 1–6) are easily identifiable in the 2-D gels shown in Fig. 3. All of these spots are expressed at a relatively higher level than the total protein obtained from wounded Chinese cabbage leaves (Fig. 3B). We used the proteomics method described above to identify the spots (Fig. 3B). The three proteins represented by spots 1, 3, and 6 are heat shock cognate protein HSC70, polygalacturonase, and peroxidase P7, respectively (Table 2). These proteins are likely to play crucial roles in wounding-stimulated defense mechanisms. However, the functions of the remaining proteins, lysine-ketoglutarate reductase/saccharopine dehydrogenase, SLG12, and efflux carrier, in plant defense are unclear (Table 2).

Discussion

Defense-related Chinese cabbage proteins induced by SA or wounding were identified using a proteomic technique. We first demonstrated that this technique was useful for identifying PR1a as a protein that is induced by SA. We then used this technique to identify other defense-related proteins that are induced by SA treatment or wounding.

Total proteins prepared from SA-treated Chinese cabbage leaves were separated by 2-D gel electrophoresis. A protein spot of interest was digested in the gel with trypsin, its peptide mass fingerprint was acquired, and identified as PR1a (Asterisk in Fig. 1). The results of Northern blot analysis also indicated that PR1a mRNA is induced by SA or wounding (Fig. 2). This experiment demonstrates that our proteomic approach should be a powerful tool for characterization of defense-related Chinese cabbage proteins.

Among the seven SA-induced proteins identified, four may be defense-related: defense-related protein, PLD, resistance protein RPS2 homolog, and L-ascorbate peroxidase (Table 1). Recent studies have begun to elucidate the signals produced by PLD, which include activation of a MAP kinase cascade, triggering of oxidative burst by phosphatidic acid and conversion of linolenic acid into bioactive octadecanoids such as JA, a known regulator of plant defense responses (Lee et al., 1997; Ryu and Wang, 1998; Laxalt and Munnik, 2002). RPS, which is encoded by an *Arabidopsis* R (resistance) gene, contains an LRR motif that participates in the protein-protein interactions that regulate pathogen-related defense pathways (Moffett et al., 2002). Chinese cabbage RPS2 also has an LRR motif that appears to regulate plant defense mechanisms. H_2O_2 has a novel function in disease resistance as a second messenger for SAR mechanisms in which SA is required for response induction (Alvarez et al., 1998). Intracellular H_2O_2 accumulation activates H_2O_2-scavenging enzymes such as catalase and peroxidase in both animals and plants (Chen et al., 1995; Berlett and Stadtman, 1997; Henle and Linn, 1997). Even though SA is required for H_2O_2-induced SAR, it inactivates catalase that elicits deleterious effects on plant health (Takahashi et al., 1997). Removal of intracellular H_2O_2 by other H_2O_2-scavenging systems such as peroxidase may therefore be more desirable. Our result that SA induces L-ascorbate peroxidase suggests that peroxidase may be involved in this process (Table 1 and Fig. 1). Interestingly, wounding also induced expression of peroxidase P7 protein (Table 2), suggesting that this peroxidase may be required for wounding-induced defense responses. Although our results demonstrate that SA evokes induction of defense-related protein, PLD, resistance protein RPS2 homolog, and L-ascorbate peroxidase at the protein level, their precise functions in plant defense responses need to be studied further. The functions of other SA-induced proteins including arginase, S-locus related 2-9A, and protein serine/threonine kinase BNK1 in plant defense responses are not known (Table 1); further studies of these proteins should provide clues about their role in plant defense.

Among the six wounding-induced proteins identified, HSC70, polygalacturonase, and peroxidase P7 may be defense-related (Table 2), while functions of lysine-ketoglutarate reductase/saccharopine dehydrogenase, SLG12, and efflux carrier in wounding-related defense responses are not known (Table 2). Although the precise role of HSC70 in plant wounding-related defense

responses has not yet been elucidated, several studies have demonstrated that it is induced by pathophysiological stresses such as oxidants, injury, and viral and bacterial infection, as well as by physiological stress such as heat shock (Sorger, 1991; Morimoto et al., 1992; Zhang and Glaser, 2002). The hsp70 genes have been cloned from a wide variety of organisms, including yeast, insects, tomato, mouse, and humans. Our finding that HSC70 is induced by wounding in Chinese cabbage leaves indicates that plants may produce HSC70 during defense signaling in the absence of pathogens. Polygalacturonase (pectinase) may play a crucial role in pectin degradation. During pathogenesis, the attacking pathogens secrete polygalacturonase, which degrades the cell wall (Pear et al., 1993). Pectic fragments released from the plant cell wall then act as a signal that activates a defense response against the pathogens (Farmer et al., 1991; Bergey et al., 1996). The observation that wounding induces polygalacturonase suggests that Chinese cabbage can produce pectic fragments during defense signaling regardless of the type of pathogen. Despite our identification of HSC70, polygalacturonase, and peroxidase P7 as wounding-inducible proteins, the biological activities of these proteins must be characterized to gain a clear understanding of their roles in Chinese cabbage defense response.

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