Transgenic expression of cecropin B, an antibacterial peptide from *Bombyx mori*, confers enhanced resistance to bacterial leaf blight in rice

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Received 27 July 2000; revised 4 September 2000; accepted 21 September 2000

Edited by Pierre Jolles

Abstract The short persistence of cecropin B peptide in plants, due to post-translational degradation, is a serious impediment in its effective utilization for developing bacterial resistance transgenic plants. Two DNA constructs encoding the full-length precursor of cecropin B peptide and the mature sequence of cecropin B peptide preceded by a signal peptide derived from rice chitinase gene were transformed in rice. The differences in the transcriptional levels in independent transgenic lines showed moderate to high expression of cecropin B gene that correlated well with the differences in cecropin B accumulation observed by Western blot analysis. The development of lesions resulting from infection by *Xanthomonas oryzae* pv. *oryzae* was significantly confined in the infected leaflet of transgenic lines, when compared with the control plants.

Key words: Antibacterial peptide; Cecropin-transgenic rice; Signal peptide; Disease resistance; Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*)

1. Introduction

Over the last few decades, use of agrochemicals in controlling phytobacterial attack has played a major role in meeting world food needs. However, in recent years, concerns have arisen out of findings relating these agrochemicals to adverse impact on human health and environment. Likewise, conventional plant breeding has been considerably successful in the development of new plant cultivars with enhanced resistance to several diseases, but due to the paucity of genes for these traits in the usable gene pools, this approach has limited scope. Genetic engineering of plants by transferring a gene of interest in otherwise superior genotypes has now been accepted as a method of choice for directional improvement. To engineer plant resistance against bacterial diseases, different genetic strategies have been proposed which also include antibacterial proteins of non-plant origin (see review [1]).

Cecropins are a family of potent antibacterial peptides in immune hemolymph of the *Cecropia* moth [2]. They are synthesized in the fat bodies as a preproprotein consisting of 31–39 amino acid residues and adapt α-helical structure on interaction with bacterial membranes resulting in the formation of ion channels [3]. At low concentrations (0.1–5 μM range), cecropins exhibit lytic antibacterial activity against several Gram-negative and Gram-positive bacteria, but not eukaryotic cells [4,5], thus making it a potentially powerful tool for engineering bacterial disease resistance into crops.

The introduction of genes encoding small antimicrobial peptides when introduced into plants did enhance their resistance to bacterial and fungal pathogens [6–9]. Also, genes encoding cecropins and its analogs have been expressed in transgenic tobacco with contradictory results regarding pathogen resistance. Reduced disease symptoms upon bacterial infection were observed in transgenic tobacco expressing cecropin analogs [10,11]. In contrast to these results, no difference in disease response was observed in transgenic tobacco/potato plants expressing the genes for either the cecropin B or a chimeric cecropin B/cecropin A [12–14]. Subsequent studies revealed that expression of cecropin B gene in plants does not result in detectable cecropin B levels, presumably due to degradation of the peptide by host peptidases [15]. Protection of cecropin B from cellular degradation was, therefore, expected to provide a viable approach towards developing bacterial resistance transgenic plants.

We have previously isolated and characterized cecropin B from *Bombyx mori* [16] and evaluated its in vitro biological activity against several phytopathogenic bacterial species [17]. In this study, we report for the first time that cecropin B peptide when translocated into the intercellular spaces in rice transgenic plants is protected from the degradation by plant peptidases and confers enhanced resistance against *Xanthomonas oryzae* pv. *oryzae* infection.

2. Materials and methods

2.1. Construction of expression vectors

For transformation, two expression vectors were constructed that contain cecropin B gene from *B. mori* driven by the control of a high expression promoter, $E_7\Omega$In [18].

Plasmid 1 (pRcec19-1). A DNA fragment (~190 bp) containing the complete coding region of cecropin B precursor (*cecB*) was amplified from a cDNA clone, BmCec19 [16], that served as a template in a PCR amplification to create *Bam*HI and *Sac*I sites by using gene-specific primers Cec19a GTAC*ggatcc*GCTTGTGTCTTAACG and Cec19b AAA*gagctc*TTTCCGATAGCTTTAGCCG (small, italicized letters in the primer denote a restriction enzyme site for subcloning of the DNA fragment containing cecropin B gene). The cecropin B gene fragment obtained was ligated into *Bam*HI/*Sac*I-digested pBI7133 [18] to generate the pRcec19-1 gene construct (Fig. 1).

Plasmid 2 (pRSPcec19-2). In order to secrete cecropin B intercellularly, signal peptide of the rice chitinase protein was fused with the

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PII: S0014-5793(00)02106-2

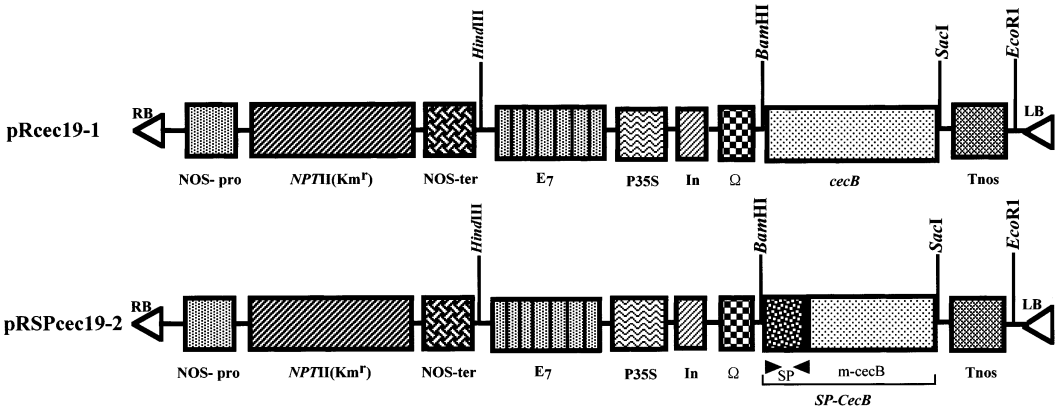

Fig. 1. Schematic representation of cecropin B gene constructs driven by a high expression promoter vector, $E_7\Omega In$, which includes tandem repeats of the 5′ enhancer sequences of the CaMV 35S promoter, omega (Ω) sequence from the tobacco mosaic virus and the first intron of a gene for phaseolin, used for rice transformation. pRcec19-1 encodes the full-length cecropin B precursor (*cecB*). pRSPcec19-2 encodes the mature sequence of cecropin B (m-cecB) peptide preceded by a signal peptide (SP) derived from rice chitinase gene (*SP-cecB*). The T-DNA of these binary vectors has the *npt*II gene under the control of NOS promoter for a selectable marker, which confers resistance to the antibiotic kanamycin. Tnos is polyadenylation signal of the CaMV 35S transcript.

coding sequence of mature peptide of cecropin B (*SP-cecB*). For this, a signal peptide coding sequence of rice chitinase gene (RCG3) [19] was amplified using primers Cht-1 CATT*ggattcc*CATTGCCGGCC-GCCATGAGAGCACTCGC (small, italicized letters in the primer denote a restriction enzyme site) and Cht-2 GATCTTCCACC-TGCCGCGCACGGC. The primer Cht-2 is an overlapping primer, which contains a part of the cecropin B coding sequence (underlined) and a partial signal peptide sequence (double underlined) from the rice chitinase gene. The DNA fragment containing the coding sequence of mature cecropin B was amplified by using primers Cec19c AGGTGGAAGATCTTCAAGAAAATT (underlined letters denote complementary sequence of cecropin B mature peptide with the underlined letters in Cht-2 primer) and Cec19b. Both fragments were directly joined by recombinant PCR in frame using *Pfu* DNA polymerase [20]. The authenticity of the PCR-amplified fragment from the chimeric product was confirmed by sequencing and ligated into *Bam*HI/*Sac*I-digested pBI7133 [18] to generate the pRSPcec19-2 gene construct (Fig. 1). The resulting binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by a freeze–thaw method.

2.2. Transformation and regeneration of transgenic plants

Stable transgenic lines of rice (*Oryza sativa* L. *japonica* cv. Nipponbare), with pRcec19-1 and pRSPcec19-2 gene constructs were developed following the protocol by Hei et al. [21]. About 20–25 kanamycin resistant plants generated for each construct were grown in individual pots in a growth chamber under a 16 h light/8 h dark regime. Integration of constructs into the rice genome was confirmed by PCR analysis. The progeny (T_1) was used to determine the expression and resistance level in the transgenic rice plants.

2.3. Expression of cecropin B in transgenic plants

Total RNA was isolated from leaf tissues of 5–6 week old plants using RNeasy Plant Mini kit (Qiagen). For Northern analysis, total RNA was denatured in formaldehyde, electrophoresed and transferred to a Hybond™-N^+ nylon membrane (Amersham Pharmacia Biotec, UK). Hybridization and washing of the blots were done using standard protocols.

2.4. Bacterial isolate, inoculation and resistance scoring in transgenics

X. oryzae pv. *oryzae* isolate was grown for 72 h at 30°C on peptone sucrose agar. The bacterial suspension for inoculation was prepared by adding sterile distilled water to the surface of the agar media and adjusted the bacterial population to 1×10^8 colony forming units per ml. Six week old, green house grown rice plants (T_1) with fully expanded leaves were wounded with multiple needles [22]. After inoculation, plants were kept in a growth chamber. The size of the lesion on the leaves was measured to evaluate the level of resistance by scoring the appearance of bacterial leaf blight symptoms for 12 days after inoculation. Scoring was done based on a disease index using a five-point scale as described by Ou et al. [22].

2.5. Protein extraction and Western blot analysis

Protein extraction from control and transgenic rice plants was performed using extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 20 mM sodium metabisulfate, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA), 1 μM pepstatin (Sigma) and 0.5 μg/ml leupeptin). The apoplastic fluid from rice leaves was collected by centrifugal extraction of intercellular fluid [23] and the remaining leaf tissues were extracted to obtain the intracellular protein.

For Western blotting, fixed amounts of total protein extracts were run on Tricine–SDS–PAGE [24] and transferred onto a PDVF nylon membrane (Amersham Pharmacia Biotec, UK). Immunodetection was performed using polyclonal rabbit anti-cecropin B serum as primary antibody with a 1:3000 dilution of goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Amersham Pharmacia Biotec). Detection of antigen–antibody complexes was performed with enhanced chemiluminescence using ECL™-Plus kit (Amersham Pharmacia Biotec), and the images were recorded on X-ray film.

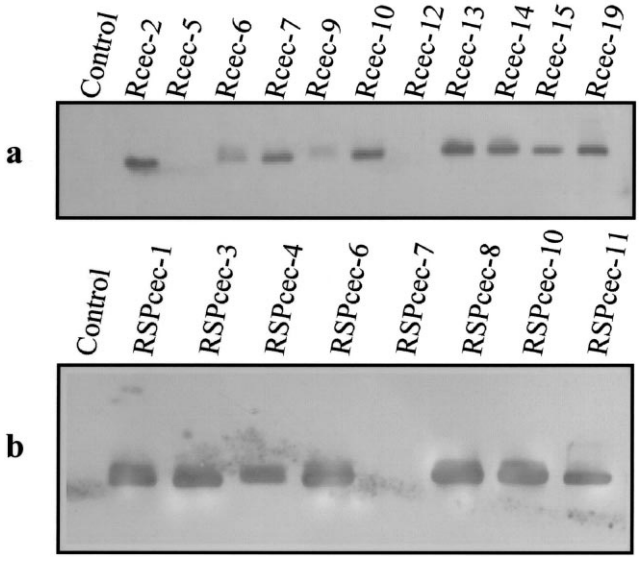

Fig. 2. Autoradiographs of Northern blot analysis of total RNA isolated from selected transgenic rice plants. Transgenic plants with pRcec19-1 (Rcec-2, -5, -6, -7, -9, -10, -12, -13, -14, -15, -19) or with pRSPcec19-2 (RSPcec-1, -3, -4, -6, -7, -8, -10, -11) were analyzed.

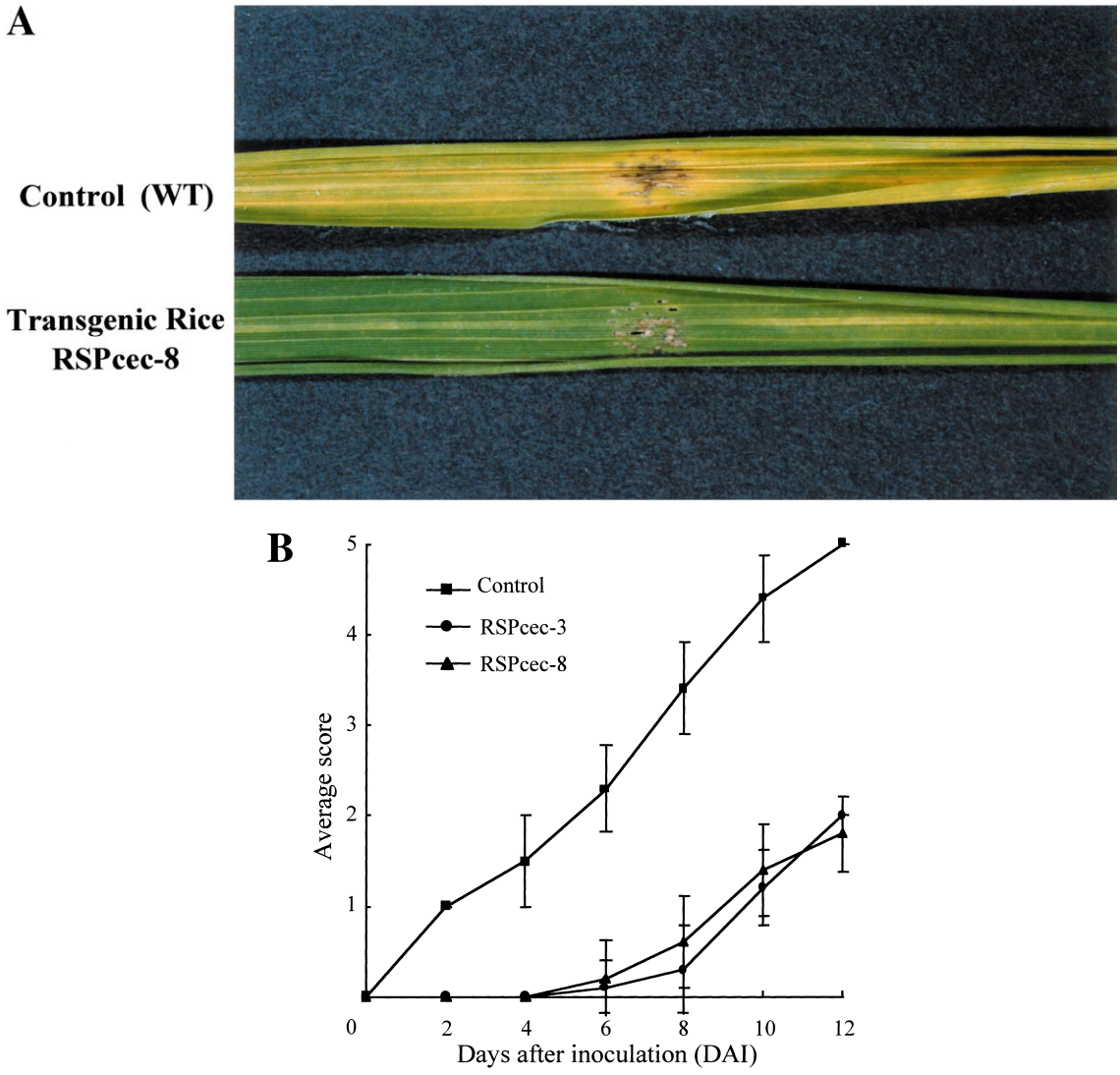

Fig. 3. Enhanced resistance to bacterial leaf blight disease in cecropin B-transgenic rice plants. (A) Disease symptoms caused by *X. oryzae* pv. *oryzae* on detached leaflet of rice transformed with pRSPcec19-2 gene construct (*SP-cecB*) and untransformed control lines. Transgenic (RSPcec-8) and control (WT) rice leaves were inoculated and evaluated as described in Section 2. Photograph was taken 10 days after inoculation showing confined lesions growth in RSP-8 as compared to WT with coalescent and elongated lesions. (B) Progression of *X. oryzae* pv. *oryzae* infection in transgenic (RSPcec-3 and RSPcec-8) and control rice plants. Points represent the mean of six replicate inoculations of each line. Vertical bars represent standard errors of the mean.

3. Results

3.1. Expression of cecropin B gene constructs in rice

Seeds from the transgenic plants transformed with either pRcec19-1 (*cecB*) or pRSPcec19-2 (*SP-cecB*) were germinated in the presence of kanamycin, and a 3:1 segregation for resistance to this antibiotic was observed in most of the progenies indicating a single copy insertions. The presence of a ~190 bp reverse transcription PCR product in rice transformants which was absent in RNA from untransformed plants was further analyzed to determine the level of cecropin B mRNA transcript by Northern blot analysis for both the constructs (Fig. 2). Hybridization with a cecropin B-specific probe indicated that the level of cecropin B mRNA varied in different transgenic plants and also depended on the constructs used in the transformation. As shown in Fig. 2, the expression level of cecropin B transcript was significantly more in transgenic plants containing the *SP-cecB* gene (pRSPcec19-2) as compared to the construct with only *cecB* gene (pRcec19-1).

3.2. Resistance of cecropin B-transformed rice to bacterial leaf blight

Because cecropin B showed 50% growth inhibition of *X. oryzae* pv. *oryzae* at a concentration of 1.8 ± 0.2 μg/ml in vitro growth inhibitory assay carried out by our group previously [17], we assessed the resistance of cecropin B-transformed plants to the bacterial leaf blight using both the detached leaflet assay and the whole-plant infection test. Transgenic plants from the progenies of transformants Rcec-2 and Rcec-13, which had the highest expression levels among those carrying the pRcec19-1 gene construct (*cecB*), as well as from the progenies of regenerants RSPcec-3 and RSPcec-8, which carried the pRSPcec19-2 construct (*SP-cecB*), were used for the experiment. The symptoms caused by *X. oryzae* pv. *oryzae* on detached leaflets of RSPcec-3- and RSPcec-8-

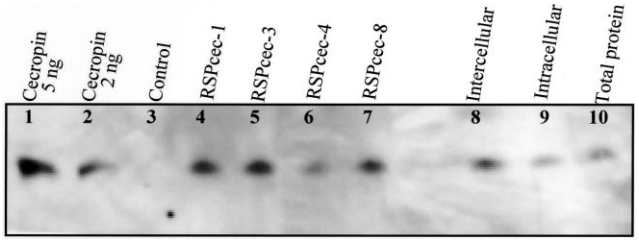

Fig. 4. Western blot analysis of control and transgenic rice lines transformed with pRSPcec19-2 gene construct (*SP-cecB*). Lanes 1 and 2: synthetic cecropin B, 5 ng and 2 ng, respectively. Lane 3: ICF of untransformed rice. Lanes 4–7: ICF of independent transgenic lines transformed with *SP-cecB*. Lanes 8–10: ICF, intracellular and total soluble protein of transgenic line RSPcec-8.

transformed lines progressed more slowly than on leaflets of the Rcec-2, Rcec-13 and control lines. This was reflected by the reduced growth rate of lesions from the infected leaflets of the transgenic lines obtained through only pRSPcec19-2. The lesions caused by the pathogen are exemplified in Fig. 3A and the quantitative results are summarized in Fig. 3B. It is evident that the appearance of the lesions on leaflets of transgenic lines RSPcec-3 and RSPcec-8 was confined only to the site of inoculation even after 10 days of inoculum application as compared to the non-transgenic control plants which were susceptible to this pathogen and showed coalescent and expanded lesions. Transgenic lines that were transformed with the pRcec19-1 construct did not show significant protection against *X. oryzae* pv. *oryzae* infection (data not shown).

3.3. Gene expression and intercellular localization of cecropin B in transgenic rice

To determine the level of cecropin B gene product produced in transgenic rice plants obtained by pRcec19-1 and pRSPcec19-2, Western blot analysis was performed using the antibody raised against the cecropin B peptide. A specific signal of the 4 kDa protein band was detected in most of the transgenic plants containing the pRSPcec19-2 gene construct (Fig. 4). However, no specific signal could be detected in pRcec19-1 introduced plants (data not shown).

Because the chimeric cecropin B gene (pRSPcec19-2) introduced into rice plants contained chitinase signal peptide sequence, the intercellular targeting of cecropin B in transgenic rice lines was examined. As shown in Fig. 4, Western blot analysis of the protein from the intercellular wash fluid (ICF) of leaves showed that the majority of cecropin B is secreted into the apoplast (lanes 4–7), though lower amounts of cecropin B were also detected in the intracellular fractions (lane 9). The minor difference in the movement of protein band detected from total leaf protein (lane 10) may represent cecropin B at different states of processing. The differences in the transcriptional and translational levels revealed by Northern (Fig. 2b) and Western (Fig. 4) blot analysis, respectively, were in good agreement for most of the lines.

4. Discussion

Earlier attempts made towards developing transgenic plants using cecropin B gene did not meet with success because of cellular degradation of this peptide by plant endogenous peptidases, thereby limiting a critical level sufficient to kill the pathogen [12–14]. Interestingly, the rate of degradation of cecropin B and its structural analog varied in intercellular fluids of different crops. Owens and Heutte [25] showed the half-life of cecropin B peptide to be 3 min in potato, 2.5 h in tobacco and 25.5 h in rice. Similar results were obtained in our laboratory indicating enhanced stability of cecropin B in rice intercellular fluid when compared to tobacco (unpublished data). These findings strongly suggested the possibility of tailoring cecropin B protein genes for plants showing a reduced rate of protein degradation. In order to make this protein effective for the control of bacterial leaf blight in rice, it was imperative that this peptide must be translocated into the intercellular spaces at the earliest to prevent cellular degradation. This was achieved by fusing the coding sequences of mature protein of cecropin B to the sequence of signal peptide of chitinase gene of rice which is known to direct the secretion of chitinase gene product into the intercellular spaces in rice [19]. In fact, signal peptide of PR1a gene of tobacco for expressing sarcotoxin 1A peptide has been successfully used in tobacco for developing resistance to bacterial disease [9]. The transgenic rice plants developed in the present study carrying cecropin B gene fused with signal peptide sequence of chitinase gene differed significantly in their reaction to *X. oryzae* pv. *oryzae*. While plants carrying *SP-cecB* gene (pRSPcec19-2) showed reduced growth of the lesions and thereby better protection from the pathogen, the transgenics having only *cecB* gene (pRcec19-1) were found to be susceptible. The increased resistance manifested by the reduced size of the lesions was observed in all the transgenic lines harboring *SP-cecB* gene construct. In transgenic plants with relatively low levels of *SP-cecB* gene expression, bacterial growth though significantly reduced, but was not completely arrested. Interestingly, the signal peptide used in the present study also enhanced the level of transcription of cecropin B gene. This is a significant observation because the elevated levels of transcription will add to the available levels of cecropin B in the intercellular spaces. The results from the Western blot analysis correlated well with those from *X. oryzae* pv. *oryzae* susceptibility test. In particular transgenic plants RSPcec-3 and RSPcec-8 which showed a high level of resistance (Fig. 3), also showed the highest level of intercellular cecropin B peptide in rice plants (Fig. 4) where it was protected from cellular degradation and could effectively contain the invading pathogen. Taken together, the results obtained in the present study are quite encouraging and unambiguously suggest that constitutive expression of peptidase sensitive cecropin B like lytic peptides when attached to the signal peptides for their translocation into intercellular spaces provides an exciting approach for effectively utilizing them in developing bacterial resistant transgenic crop plants. Further efforts are in progress to optimize the expression of more active defense genes to test the generality of this study.

Acknowledgements: We would like to thank Drs. Y. Ohashi and Y. Nishizawa for providing $E_7\Omega$In and RCG3, respectively. This work was partly supported by Enhancement of Center for Excellence, special Coordination Funds for Promoting Science and Technology to M.Y. A.S. is a recipient of a STA fellowship administered through Japan International Science and Technology Exchange Center.

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