Prevention of Copper-Induced Calcium Influx and Cell Death by Prion-Derived Peptide in Suspension-Cultured Tobacco Cells

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Impact of copper on the oxidative and calcium signal transductions leading to cell death in plant cells and the effects of the copper-binding peptide derived from the human prion protein (PrP) as a novel plant-protecting agent were assessed using a cell suspension culture of transgenic tobacco (*Nicotiana tabacum* L., cell line BY-2) expressing the aequorin gene. Copper induces a series of biological and chemical reactions in plant cells including the oxidative burst reflecting the production of reactive oxygen species (ROS), such as hydroxyl radicals, and stimulation of calcium channel opening, allowing a transient increase in cytosolic calcium concentrations. The former was proven by the action of specific ROS scavengers blocking the calcium responses and the latter was proven by an increase in aequorin luminescence and its inhibition by specific channel blockers. Following these early events completed within 10 min, the development of copper-induced cell death was observed during additional 1 h in a dose-dependent manner. Addition of a synthetic peptide (KTNMKHMA) corresponding to the neurotoxic sequence in human PrP, prior to the addition of copper, effectively blocked both calcium influx and cell death induced by copper. Lastly, a possible mechanism of peptide action and future applications of this peptide in the protection of plant roots from metal toxicity or in favour of phytoremediation processes are discussed.

Key words: Aequorin, Copper Phytotoxicity, Prion Protein

Introduction

Induction of cell death by oxidative stresses accompanying the generation of reactive oxygen species (ROS) is often mediated by early signaling events such as calcium influx *via* ROS-mediated activation of calcium channels on the plasma membrane (Kadono *et al.*, 2006). Our previous studies have revealed that various phytotoxic metals such as Al and 15 rare earth elements induce an acute generation of ROS, such as superoxide anion radicals, by stimulating the plant NADPH oxidase activity (Kawano *et al.*, 2001, 2002, 2003) and, as a consequence, ROS stimulates the opening of ROS-responsive calcium channels on the surface of plant cells (Kawano *et al.*, 2003, 2004). Copper is also known to be a phytotoxic metal which induces an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_c$) in cultured tobacco cells (Inoue *et al.*, 2005). However, the possible mechanism of acute copper action may differ from such metals stimulating the superoxide generation as production of superoxide could not be detected after addition of $CuSO_4$ to a plant cell suspension culture (Kawano and Muto, 2000). On the other hand, copper may catalyze the Fenton-type reaction forming hydroxyl radicals (the most violent members of ROS) in the presence of both certain reducing agents (such as ascorbate), converting Cu(II) to Cu(I), and biologically supplied hydrogen peroxide. Previously, monitoring of the Fenton-type reaction leading to the formation of hydroxyl radicals by electron spin resonance spectroscopy was performed and this reaction was shown to proceed in a tobacco BY-2 cell suspension culture after addition of $CuSO_4$ (Kawano and Muto, 2000).

Recently, we have been engaged in designing artificial peptides for the protection of plant cells from various metal stresses. Since our recent reports suggested that the human prion protein (PrP)-derived copper-binding peptides function as both chelators of copper and rare earth elements (Kawano, 2006) and catalysts for ROS generation (Kawano, 2007), we expected that these peptides could be used as positive and/or negative modulators of copper toxicity in plant cells. Among four distinct Cu-binding peptides derived from human PrP, only the peptide KTNMKHMA, corresponding to the partial sequence within the neurotoxic region, was shown to lack the pro-oxidant activity while other three peptides catalyzed the robust production of ROS in the presence of some biological components (Kawano, 2007; Yokawa *et al.*, 2009). Therefore, we expected that the KTN-MKHMA peptide behaves as an antioxidant with copper-chelating activity rather than being a pro-oxidant.

Among the peptides or proteins of foreign origins drastically affecting the fate of plant cells, we can name some proteinaceous elicitors known to be secreted from pathogenic microbes as well studied examples. Harpins are typical for such proteins which are heat-stable, glycine-rich type III secreted proteins produced by Gram-negative plant pathogenic bacteria such as *Erwinia amylovora* (secretion mechanisms are widely preserved), that cause a hypersensitive response when applied to the cells of tobacco (Wei *et al.*, 1992; Galan and Collmer, 1999), and *Arabidopsis thaliana* (Reboutier *et al.*, 2005, 2007), possibly through regulation of ion channels (El-Maarouf *et al.*, 2001). Recent studies have pointed out that harpin proteins (particularly the motif 2 region) that interact with host plant cells have great similarity with PrP in their capability of fibril formation (Oh *et al.*, 2007; Resende *et al.*, 2003) and in the structure (highly homologous to the prion-forming domain within the yeast prion protein Rnq1p). Due to similarity with toxic plant proteins, we can expect that artificial application of PrP or peptides derived from it may have certain impacts on plant life.

In the present study, we examined the effect of a copper-binding peptide derived from PrP on the toxic action of copper (especially calcium signaling and cell death responses) in suspension-cultured tobacco BY-2 cells expressing the calcium-sensitive luminescent protein aequorin.

Material and Methods

Plant material

A tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell suspension culture (cell line BY-2) expressing apoaequorin exclusively in the cytosol was propagated as previously reported (Takahashi *et al.*, 1997). Briefly, the culture was maintained in Murashige-Skoog (MS) liquid medium (pH 5.8) containing 0.2 μg/ml of 2,4-dichlorophenoxy acetic acid at 23 °C in the darkness while shaking on a gyratory shaker and subcultured every 14 d with a 3% (v/v) inoculum. The cells harvested 5 d after subculturing were used for the experiments.

Coelenterazine

Coelenterazine, a luminophore required for the reconstitution of aequorin from apoaequorin (Shimomura and Johnson, 1978), was chemically synthesized according to Isobe *et al.* (1994).

Peptide synthesis

As shown in Fig. 1A, in mammalian PrPs, seven copper-binding sites consisting of four distinct amino acid sequences are found, namely: (1) the four time-repeated octarepeat regions, (2) a short sequence immediately following, (3) the neurotoxic region, and (4) the helical Cu-binding region. The peptide corresponding to the neurotoxic region and chemically synthesized and purified by high pressure liquid chromatography was KTNMKHMA (purity, 98.85%); it was obtained from the custom peptide service department of Sigma Genosys Japan (Ishikari, Hokkaido, Japan). Fig. 1B shows the possible structure of the Cu-loaded KTNMKHMA peptide based on the rule proposed by Fang *et al.* (2004, 2006).

Treatments

CuSO$_4$ was first dissolved in water. Onto the cell suspension in MS medium (390 μl), the copper solution (0.35 mM final concentration) was added. When required, the peptide was mixed up with the copper solution prior to the addition to the cell suspension.

Monitoring of $[Ca^{2+}]_c$

The changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) were monitored by Ca^{2+}-dependent emission of blue light from aequorin
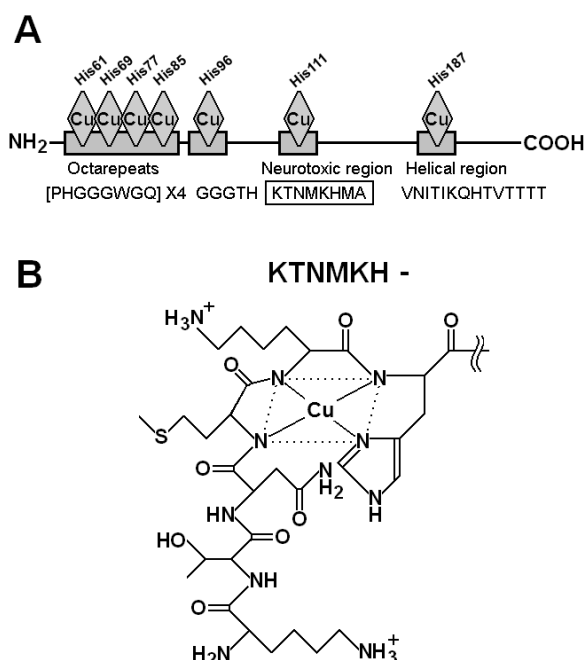
A

B KTNMKH -

Fig. 1. Copper-binding sites in human PrP. (A) Known Cu(II)-binding sites, corresponding amino acid sequences, and position numbers of anchoring His residues in human PrP. (B) Possible structure of Cu-bound peptide. The complex structure was estimated according to the works by Fang *et al.* (2004, 2006) on Ni- and Cu-binding peptides.

as previously described (Kawano *et al.*, 1998). The active form of aequorin was reconstituted by addition of 1 μM coelenterazine to the suspension culture of apoaequorin-expressing tobacco cells, 8 h prior to the measurements of $[Ca^{2+}]_c$. The aequorin luminescence was measured using a CHEM-GLOW photometer (American Instrument Co., Silver Spring, MD, USA), equipped with a pen recorder and a luminometer (Luminescensor PSN AB-2200-R, Atto Corp., Tokyo, Japan), and expressed as relative luminescence units (rlu). The traces of $[Ca^{2+}]_c$ signatures were obtained mostly using the former equipment and kinetic analyses were mostly carried out using the latter equipment.

Determination of cell death

Confluent cultures maintained at 7-day-intervals of sub-culturing were used to inoculate the fresh MS liquid medium (1 ml culture to 30 ml

medium) and precultured for 5 d. These 5-day-old cultures were harvested and used for the experiments.

Copper-induced cell death in the cell suspension culture was allowed to develop in the presence of the cell death staining dye Evans blue (0.1%, w/v). Evans blue was added to the cell suspension culture, following 10 min of calcium measurements after Cu application. Cells were further incubated for 1 h in order to fully develope and detect the cell death as described by Kadono *et al.* (2006). After terminating the staining process by washing, stained cells were counted under microscopes. For statistical analyses, 4 different digital images of cells under the microscope (each covering 50 cells to be counted) were acquired, and stained cells were counted.

Results and Discussion

Addition of copper resulted in a rapid and transient increase in $[Ca^{2+}]_c$

Aequorin was reconstituted from apo-protein, expressed in the cytosol of tobacco BY-2 cells, by adding coelenterazine to the culture 8 h prior to the experiments. Addition of $CuSO_4$ (from 0.03 μM to 10 mM) resulted in a rapid and transient increase in aequorin luminescence reflecting the acute increase in $[Ca^{2+}]_c$ (Figs. 2A, B). Compared to the positive control for the Cu (0.35 mM)-induced increase in aequorin luminescence, the cells pretreated with 1.25 mM dimethylthiourea (DMTU), a known scavenger of hydroxyl radicals, and two calcium channel blockers ($LaCl_3$ and $AlCl_3$, 5 mM and 2.5 mM, respectively) showed significant inhibition of the Cu-dependent $[Ca^{2+}]_c$ increase (Figs. 2B, C). Therefore, the Cu-induced increase in aequorin luminescence can be attributed to the influx of extracellular Ca^{2+} into the cytosolic space through activation and opening of calcium channels of the plasma membrane, which are responsive to hydroxyl radicals. However, addition of catalase (1000 units) was not effective enough to prevent the plant cells' response to the addition of copper (Fig. 2C), suggesting that Cu may penetrate deeper into the sites of the apoplastic cavity or intracellular space where macromolecules such as catalase could not reach. In this way, we understood the possible action mode for smaller scavenging molecule, such as DMTU.
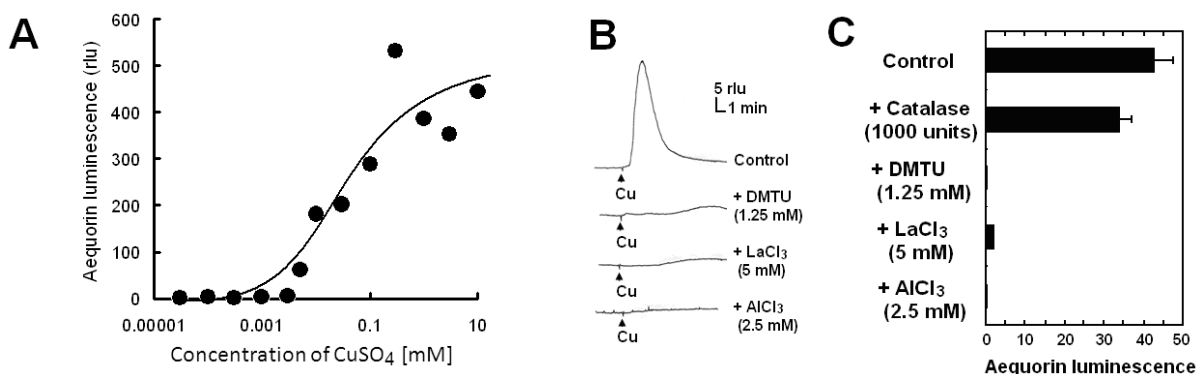
Fig. 2. Effects of ROS scavengers and calcium channel inhibitors on the Cu-induced $[Ca^{2+}]_c$ elevation in tobacco BY-2 cells expressing aequorin. (A) Effect of $CuSO_4$ concentration ranging from 0.03 μM to 10 mM on the induction of $[Ca^{2+}]_c$ increase. (B) Typical traces of Cu-induced increase in aequorin luminescence in the presence and absence of ROS scavengers and calcium channel blockers; vertical scale, relative luminescence unit (rlu). (C) Statistical analysis of the effects of various inhibitors; error bars, S.E. (n = 3, each).

Prevention of Cu-induced calcium influx by the PrP peptide

When the cells were treated with a peptide/copper mixture (*i.e.* KTNMKHMA peptide and $CuSO_4$), the Cu (0.35 mM)-induced $[Ca^{2+}]_c$ was significantly lowered by 0.15 mM peptide (Figs. 3A, B). The Cu-induced influx of Ca^{2+} was not only inhibited but also delayed for a significant period of time. While the increase in aequorin luminescence induced in response to 0.35 mM Cu attained the peak level within 2 min of treatment, the presence of the peptide KTNMKHMA delayed the peaking time up to *ca.* 6 min, as shown in Fig. 3A (traces 3 and 4). The peptide-mediated inhibition of the copper action was shown to be dose-dependent (Fig. 3C). The above study is the first demonstration that a PrP-derived peptide sequence shows some biological actions in a plant system.

Prevention of Cu-induced cell death by peptide treatment

Following the early events represented by the ROS-mediated increase in $[Ca^{2+}]_c$ induced by Cu treatment, which are likely completed within 10 min following application of Cu, an induction of cell death was allowed for additional 1 h. Then dead cells stained by Evans blue were counted under a microscope. Data suggested that copper induces the cell death in a dose-dependent manner (Fig. 4A). As expected, addition of the synthetic peptide KTNMKHMA to the cell suspension culture, prior to addition of copper, effectively blocked the induction of cell death by copper (Fig. 4B), further confirming the plant-protective nature of the peptide acting against the Cu toxicity.

Mechanism of peptide action

The action of the PrP neurotoxic peptide KTNMKHMA against Cu is likely attributed to the chelating activity of the peptide; Fig. 1B shows the likely structure of the peptide-Cu complex. However, there must be an additional mode of peptide action as supported by the lower molar ratio of peptide over Cu required for showing the peptide action (Fig. 3). The ratio of peptide concentration over that of Cu, required for blocking the Cu action by *ca.* 80% demonstrated here, was smaller than unity, which is not high enough to be attributed solely to the chelating action of the peptide.

Recently, the antioxidative roles of the Cu-bound form of PrP^C (intrinsic cellular PrP) have been documented. It has been shown that *E. coli* cells expressing the PrP sequence (octapeptide repeats region) acquired resistance to Cu and Cu-dependent oxidative damages, indicating that PrP^C possibly contributes to the protection of cells from free Cu-catalyzed generation of ROS such as hydroxyl radicals (Shiraishi *et al.*, 2000). Watt *et al.* (2005) suggested that the Cu-bound octarepeat-dependent progress in β-cleavage of PrP^C is an early and critical event in the mechanism of protecting cells by PrP acting against the

oxidative stress. Furthermore, it was shown that Cu-bound PrP^C possesses some superoxide dismutase (SOD)-like activity *in vitro*, and its expression likely contributes to the cellular response to oxidative damages to cells (Wong *et al.*, 2001).

Sauer *et al.* (1999) have proposed that PrP^C expression in tumour spheroids is regulated by the internal redox statuses meeting the requirement to protect cells from ROS since they have observed in tumour spheroids that an increase in ROS stimulates the production of PrP^C and other ROS-scavenging enzymes such as Cu,Zn-SOD and catalase, while ROS-lowering treatments effectively down-regulate the expression of both ROS-scavenging enzymes and PrP^C.

The above studies carried out in mammalian systems are indicative of the hidden fact that the Cu-binding peptide used in our present study may exhibit additional functions upon loading of Cu, and such function may be related to the removal of ROS produced by free Cu, thus applicable for
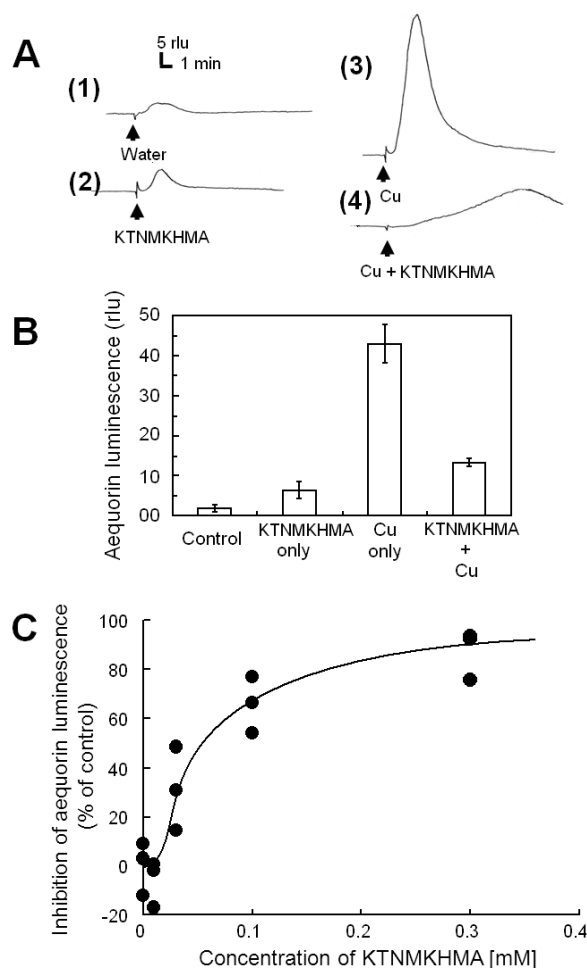

Fig. 3. Relative peak height of aequorin luminescence in tobacco BY-2 cell suspension culture. (A) Typical traces of copper-induced increase in aequorin luminescence and its inhibition by the neurotoxic PrP peptide (KTN-MKHMA); (1) water control; (2) addition of the peptide only (0.15 mM final concentration); (3) addition of CuSO$_4$ only (0.35 mM final concentration); (4) mixture of peptide (0.15 mM final concentration) and CuSO$_4$ (0.35 mM final concentration) added; vertical scale, relative luminescence unit (rlu). (B) Inhibitory effect of the peptide (KTNMKHMA) on the Cu-induced calcium influx; concentrations of the peptide and copper were identical with (A); error bars represent S.D. (*n* = 3). (C) Effect of peptide concentration on the inhibition of the Cu-induced [Ca^{2+}]$_c$ increase.
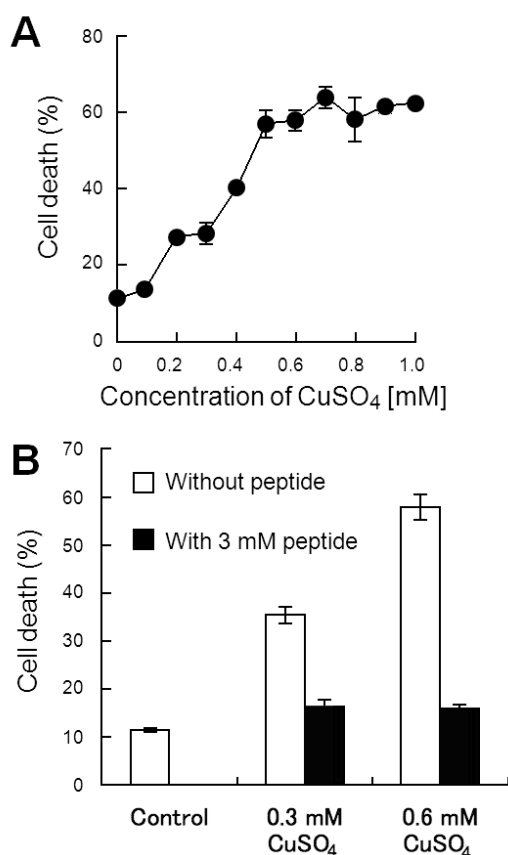

Fig. 4. Protection of tobacco BY-2 cells from copper-induced cell death by the peptide KTNMKHMA. (A) Effect of copper concentration on the induction of cell death. (B) Cell death induction by CuSO$_4$ (0.3 and 0.6 mM) and its prevention by pre-treatment with 3 mM KTNMKHMA; error bars represent S.D. (*n* = 3).

the protection of plant cells from copper toxicity. Further examinations of this hypothesis are required in future experiments, and this process may allow us to design better peptidic or biochemical agents for the protection of plant cells from metal toxicity. Since the agent tested here is a peptide, genetic modification of plants for overproduction and excretion of this or related peptidic agents is one of the possible choices in order to minimize the phytotoxicities of various metals in future environments.

Acknowledgements

El-Maarouf H., Barny M. A., Rona J. P., and Bouteau F. (2001), Harpin, a hypersensitive response elicitor from *Erwinia amylovora*, regulates ion channel activities in *Arabidopsis thaliana* suspension cells. FEBS Lett. **497**, 82–84.

Fang Y. Y., Ray B. D., Claussen C. A., Lipkowitz K. B., and Long E. C. (2004), Ni(II)·Arg–Gly–His-DNA interactions: investigation into the basis for minor-groove binding and recognition. J. Am. Chem. Soc. **126**, 5403–5412.

Fang Y. Y., Claussen C. A., Lipkowitz K. B., and Long E. C. (2006), Diastereoselective DNA cleavage recognition by Ni(II)·Gly–Gly–His-derived metallopeptides. J. Am. Chem. Soc. **128**, 3198–3207.

Galan J. E. and Collmer A. (1999), Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284**, 1322–1328.

Inoue H., Kudo T., Kamada H., Kimura M., Yamaguchi I., and Hamamoto H. (2005), Copper elicits an increase in cytosolic free calcium in cultured tobacco cells. Plant Physiol. Biochem. **43**, 1089–1094.

Isobe M., Takahashi H., Usami K., Hattori M., and Nishigohri Y. (1994), Bioluminescence mechanism on new system. Pure Appl. Chem. **66**, 765–772.

Kadono T., Yamaguchi Y., Furuichi T., Hirono M., Garrec J.-P., and Kawano T. (2006), Ozone-induced cell death mediated with oxidative and calcium signaling pathways in tobacco Bel-W3 and Bel-B cell suspension cultures. Plant Signal. Behav. **1**, 312–322.

Kawano T. (2006), Quenching and enhancement of terbium fluorescence in the presence of prion-derived copper-binding peptides. ITE Lett. **7**, 383–385.

Kawano T. (2007), Prion-derived copper-binding peptide fragments catalyze the generation of superoxide anion in the presence of aromatic monoamines. Int. J. Biol. Sci. **3**, 59–65.

Kawano T. and Muto S. (2000), Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco suspension culture. J. Exp. Bot. **51**, 685–693.

Kawano T., Sahashi N., Takahashi K., Uozumi N., and Muto S. (1998), Salicylic acid induces extracellular generation of superoxide followed by an increase in cytosolic calcium ion in tobacco suspension culture: The earliest events in salicylic acid signal transduction. Plant Cell Physiol. **39**, 721–730.

Kawano T., Kawano N., Muto S., and Lapeyrie F. (2001), Cation-induced superoxide generation in tobacco cell suspension culture is dependent on ion valence. Plant Cell Environ. **24**, 1235–1241.

Kawano T., Kawano N., Muto S., and Lapeyrie F. (2002), Retardation and inhibition of the cation-induced superoxide generation in BY-2 tobacco cell suspension culture by Zn^{2+} and Mn^{2+}. Physiol. Plant. **114**, 395–404.

Kawano T., Kadono T., Furuichi T., Muto S., and Lapeyrie F. (2003), Aluminum-induced distortion in calcium signaling involving oxidative bursts and channel regulations in tobacco BY-2 cells. Biochem. Biophys. Res. Commun. **308**, 35–42.

Kawano T., Kadono T., Fumoto K., Lapeyrie F., Kuse M., Isobe M., Furuichi T., and Muto S. (2004), Aluminum as a specific inhibitor of plant TPC1 Ca^{2+} channels. Biochem. Biophys. Res. Commun. **324**, 40–45.

Oh J., Kim J.-G., Jeon E., Yoo C.-H., Moon J. S., Rhee S., and Hwang I. (2007), Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. J. Biol. Chem. **282**, 13601–13609.

Reboutier D., Vedel R., Brault M., Duggleby R. G., Rona J. P., Barny A. M., and Bouteau F. (2005), A CFTR chloride channel activator prevents HrpN$_{ea}$-induced cell death in *Arabidopsis thaliana* suspension cells. Plant Physiol. Biochem. **43**, 567–572.

Reboutier D., Frankart C., Briand J., Biligui B., Rona J. P., Haapalainen M., Barny M. A., and Bouteau F. (2007), Antagonistic action of harpin proteins: Hrp-W$_{ea}$ from *Erwinia amylovora* suppresses HrpN$_{ea}$-induced cell death in *Arabidopsis thaliana*. J. Cell Sci. **120**, 3271–3278.

Resende C. G., Outeiro T. F., Sands L., Lindquist S., and Tuite M. F. (2003), Prion protein gene polymorphisms in *Saccharomyces cerevisiae*. Mol. Microbiol. **49**, 1005–1017.

Sauer H., Dagdanova A., Hescheler J., and Wartenberg M. (1999), Redox-regulation of intrinsic prion expression in multicellular prostate tumor spheroids. Free Radic. Biol. Med. **27**, 1276–1283.

Shimomura O. and Johnson F. H. (1978), Peroxidized coelenterazine, the active group in the photoprotein aequorin. Proc. Natl. Acad. Sci. USA **75**, 2611–2615.

Shiraishi N., Ohta Y., and Nishikimi M. (2000), The octapeptide repeat region of prion protein binds Cu(II) in the redox inactive states. Biochem. Biophys. Res. Commun. **267**, 398–402.

Takahashi K., Isobe M., Knight M. R., Trewavas A. J., and Muto S. (1997), Hypoosmotic shock induces in-

crease in cytoslic Ca^{2+} in tobacco suspension-culture cells. Plant Physiol. **113**, 587–594.

Watt N. T., Taylor D. R., Gillott A., Thomas D. A., Perera W. S., and Hooper N. M. (2005), Reactive oxygen species-mediated β-cleavage of the prion protein in the cellular response to oxidative stress. J. Biol. Chem. **280**, 35914–35921.

Wei Z. M., Laby R. J., Zumoff C. H., Bauer D. W., He S. Y., Collmer A., and Beer S. V. (1992), Harpin, elicitor of the hypersensitive response. Science **257**, 85–88.

Wong B. S., Brown D. R., Pan T., Whiteman M., Liu T., Bu X., Li R., Gambetti P., Olesik J., Rubenstein R., and Sy M. S. (2001), Oxidative impairment in scrapie-infected mice is associated with brain metals perturbations and altered antioxidant activities. J. Neurochem. **79**, 689–698.

Yokawa K., Kagenishi T., Goto K., and Kawano T. (2009), Free tyrosine and tyrosine-rich peptide-dependent superoxide generation catalyzed by a copper-binding, threonine-rich neurotoxic peptide derived from prion protein. Int. J. Biol. Sci. **5**, 53–63.