Review

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Elicitor Signal Transduction That Leads to Hypersensitive Reaction in Cultured Tobacco Cells

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Abstract

Plants have an excellent mechanism for responding to pathogen invasion by specific and inducible defense reactions, which is often apparent as the so-called hypersensitive reaction (HR). The molecular dissection of the mechanism of HR is difficult since the biological system involves intricate interactions between plant and pathogen. A simplified system, in which suspension-cultured plant cells are synchronously challenged with a single molecular inducer of HR, elicitor, would greatly facilitate studies of the molecular mechanism of HR. Several components involved in elicitor signaling have recently been identified and characterized using the experimental system.

1. Introduction

Higher plants have the ability to respond to invasion by pathogens, such as fungi, bacteria and viruses, through defense reactions that may provide protection against these pathogens. Although plants do not have specialized cells and tissues, such as cells of immune system in animals, to protect themselves against pathogen infection, they have an excellent mechanism for responding to pathogen invasion by specific and inducible defense reactions, which is often apparent as the so-called hypersensitive reaction (HR). HR resulting from the incompatible interaction between the plant and a corresponding pathogen is a multifaceted defense reaction that include phytoalexin accumulation, ion fluxes, generation of active oxygen species (i.e., oxidative burst), expression of defense-related genes, and hypersensitive cell death (Kombrink and Somssich, 1995).

The molecular dissection of the mechanism of HR is difficult since the biological system involves intricate interactions between plant and pathogen. A simplified system, in which plant cells are synchronously challenged with a single molecular inducer of HR, would greatly facilitate studies of the molecular mechanism of the HR.

In the initial step of events during defense reaction, plant cells perceive either plant-derived (endogenous) or pathogen-derived (exogenous) signals. These signal compounds are collectively referred to as elicitors (Ebel and Cosio 1994, Kombrink and Somssich 1995). The exogenous elicitors of plant defense responses differ widely in their chemical nature, and include proteins, oligosaccharides, glycoproteins and lipids. Most of the pathogen-derived elicitors identified to date are non-specific, in that they induce various defense responses in a large variety of plant cultivars and species. Inducible defense responses can be activated, not only upon challenge of plant tissues by pathogen, but also upon exposure to elicitors. Therefore, elicitors are now widely used in simple experimental systems to study the molecular mechanisms of defense responses.

The infection of plants by pathogens and the resulting defense responses occur initially at the single-cell level. Suspension-cultured cells have been widely used as a simplified experimental system for investigations of the molecular mechanisms involved in plant-pathogen interactions. Therefore, in combination with elicitors, such systems have provided excellent models for studies of various aspects of defense responses in plant cells. We have been investigating the elicitor signal-transduction pathway of plant cells, using fungal elicitors and suspension-cultured tobacco cells.

In this article, I describe current view of molecular mechanism of induced defense reaction of plant cells based on our own research and recent developments in this field.

2. Defense responses induced by fungal elicitors in tobacco cells.

To investigate an elicitor signal transduction pathway, we have been monitored early changes in morphological, biochemical and molecular events in suspension-cultured tobacco cells, line XD6S, which was derived from *Nicotiana tabacum* cv Xanthi (Yamaoka *et al.*, 1969), treated with two different types of fungal elicitors, a crude extract of cell wall of *Phytophthora infestines* (PiE) and a purified xylanase from culture of *Trichoderma viride* (TvX). As shown **Figure 1**, these two elicitors induce various kinds of defense responses, including activation of a p47 protein kinase, an oxidative burst, alkalinization of culture medium, and expression of a subset of defense-related genes (Fukuda and Shinshi, 1994; Suzuki *et al.*, 1995; Suzuki and Shinshi, 1995; Fukuda, 1996, 1997; Yano *et al.*, 1998; Suzuki *et al.*, 1999; Yano *et al.*, 1999). In addition, TvX induces shrinkage of the cytoplasm, condensation of the nucleus, and finally hypersensitive cell death (Yano *et al.*, 1998; Suzuki *et al.*, 1999; Yano *et al.*, 1999). Therefore, in this experimental system, we can investigate the specific events in hypersensitive cell death as well as general defense responses. Thus, in combination with these elicitors, XD6S cells facilitate further studies on the molecular mechanism of defense responses of plant against pathogen infection.

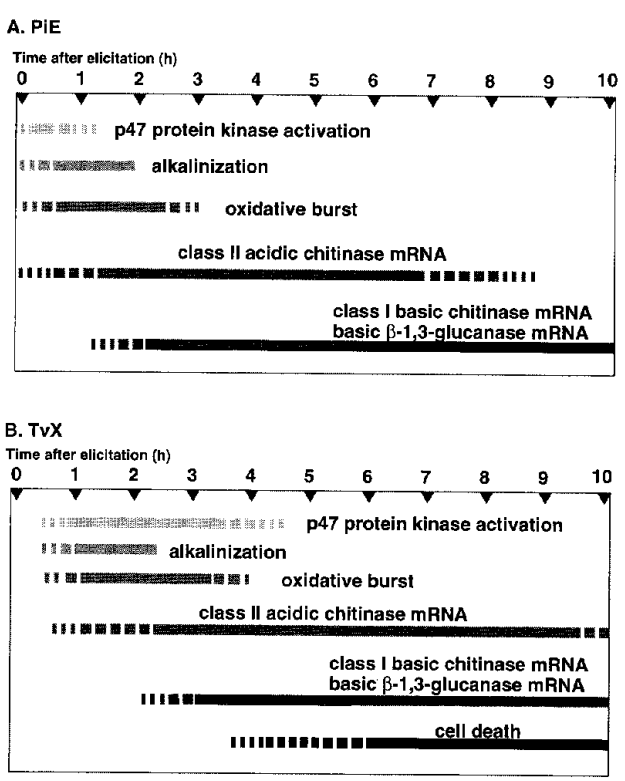

Fig. 1. Cellular events in tobacco XD6S cells that are induced by fungal elicitors, PiE (A) and TvX (B).

3. Involvement of protein kinase cascade in elicitor signal transduction.

Several lines of evidence have demonstrated that protein phosphorylation is an essential component of elicitor-induced signal transduction (Suzuki and Shinshi, 1996). Experiments with protein kinase inhibitors, such as staurosporine and K252a, have revealed that protein phosphorylation is required for induction of defense responses, including extracellular alkalinization (Atkinson *et al.* 1990, Conrath *et al.* 1991, Felix *et al.* 1991, 1993, 1994), oxidative burst (Schwacke and Hager 1992, Baker *et al.* 1993, Suzuki *et al.* 1999), ethylene production (Grosskopf *et al.* 1990, Felix *et al.* 1991), accumulation of secondary metabolites (Conrath *et al.* 1991), and accumulation of transcripts from defense genes (Raz and Fluhr 1993, Suzuki *et al.* 1995, Suzuki *et al.* 1999), and hypersensitive cell death (He *et al.*, 1994; Suzuki *et al.* 1999). In addition, elicitor treatment caused rapid changes in the phosphorylation status of characteristic proteins in microsomal and/or cytosolic preparations (Grab *et al.* 1989, Dietrich *et al.* 1990), and protein kinase inhibitors abolished elicitor-stimulated changes in protein phosphorylation (Grosskopf *et al.* 1990, Felix *et al.* 1991, 1993, 1994, Viard *et al.* 1994).

Molecular genetic studies, based on gene-for-gene complementarity in plant-pathogen interactions, have revealed that several resistance genes encode protein kinases (Martin *et al.*, 1993; Martin GB *et al.*, 1994; Song *et al.*, 1995; Zhou *et al.*, 1995). In addition, recent biochemical studies demonstrated that the hypersensitive cell death in suspension culture of soybean cells (Levine *et al.*, 1994) and of tobacco cells (Suzuki *et al.*, 1999) and in tobacco leaves (He *et al.*, 1994), induced by incompatible bacteria and elicitors can be blocked by protein kinase inhibitors. These results suggest that a protein kinase cascade is involved in the recognition of the elicitor and in the intracellular signal transduction that leads to HR including hypersensitive cell death (Bent, 1996; Suzuki and Shinshi, 1996; Jones, 1997).

4. Elicitor-responsive MAPK-like p47 protein kinase

Correlation of activity of specific protein kinase to defense responses as well as hypersensitive cell

death has rarely been demonstrated. To establish a functional link between protein phosphorylation and transduction of the elicitor signal that leads to defense responses, we have chosen a biochemical approach and attempted to analyze the elicitor-responsive protein kinase in XD6S cells, in which PiE- and TvX-induced defense responses were inhibited by staurosporine (Suzuki *et al.*, 1995; Suzuki *et al.*, 1999) and identified an elicitor-responsive 47-kD protein kinase (Suzuki and Shinshi, 1995) designated as p47 protein kinase (Suzuki *et al.* 1999). The activity of p47 protein kinase is barely detectable in untreated cells, but the kinase is activated rapidly, transiently and strongly in conjunction with its tyrosine phosphorylation, prior to defense responses, upon treatment of cells with PiE (Suzuki and Shinshi, 1995). By contrast, we found that the p47 protein kinase was slowly and extendedly activated during the TvX-induced hypersensitive cell death (Suzuki *et al.*, 1999). In addition, the activation of p47 protein kinase induced by the fungal elicitors was also inhibited by staurosporine (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1999). These findings are consistent with the model that the p47 protein kinase mediate the phosphorylation-dependent signal transduction pathway in response to fungal elicitors that leads to defense responses and hypersensitive cell death **(Fig. 3)**.

The activity of an upstream protein kinase(s) is required for the tyrosine phosphorylation and activation of the p47 kinase in response to the elicitors (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1999). In addition, anti-ERK1 antibody is bound to the p47 protein kinase (Suzuki *et al.*, 1999). Based on such biochemical features, this kinase has been postulated to be a member of the mitogen-activated protein kinase (MAPK) family (Chasan, 1995; Suzuki and Shinshi, 1995; Hirt, 1997; Mizoguchi *et al.*, 1997; Suzuki *et al.*, 1999). It has also been demonstrated that fungal and bacterial elicitors activate MAPKs and MAPK-like kinases in tobacco cells (Ádám *et al.*, 1997; Lebrun-Garcia *et al.*, 1998; Zhang *et al.*, 1998; Romeis, *et al.*, 1999). In parsley, an elicitor-responsive MAPK is probably involved in the activation of transcription of genes since it is translocated to the nucleus upon treatment of cells with elicitor (Ligterink *et al.*, 1997). Thus, an increasing body of evidence indicates that MAPK are involved in the signaling pathway that is triggered by elicitors (Suzuki and Shinshi, 1996; Ebel and Mithöfer, 1998).

Our previous results showed that different stimuli, such as transfer of cell suspension to a new plastic dish, and treatment with PiE and TvX, activate the p47 protein kinase with different kinetics followed by different responses, whereas the magnitude of activation of the p47 protein kinase was similar for these stimuli (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1999). These results suggest that the regulation of the actual duration of activation of p47 protein kinase might be crucial in the determination of subsequent responses of tobacco cell **(Fig. 2)**. Transfer of cell suspension induced very short-term and limited increase of mRNAs for genes, which were also induced by wounding in leaf tissue (Suzuki, K., Yano, A., Nishiuchi, T., and Shinshi, H., unpublished results). Therefore, it is reasonable to postulate that very short-term and limited activation of p47 protein kinase, for example by the transfer (probably mechanical) stress, is insufficient for sustained induction of downstream events in the elicitor-initiated signal transduction cascade **(Fig. 2A)**. By contrast, the elicitor-induced rapid and

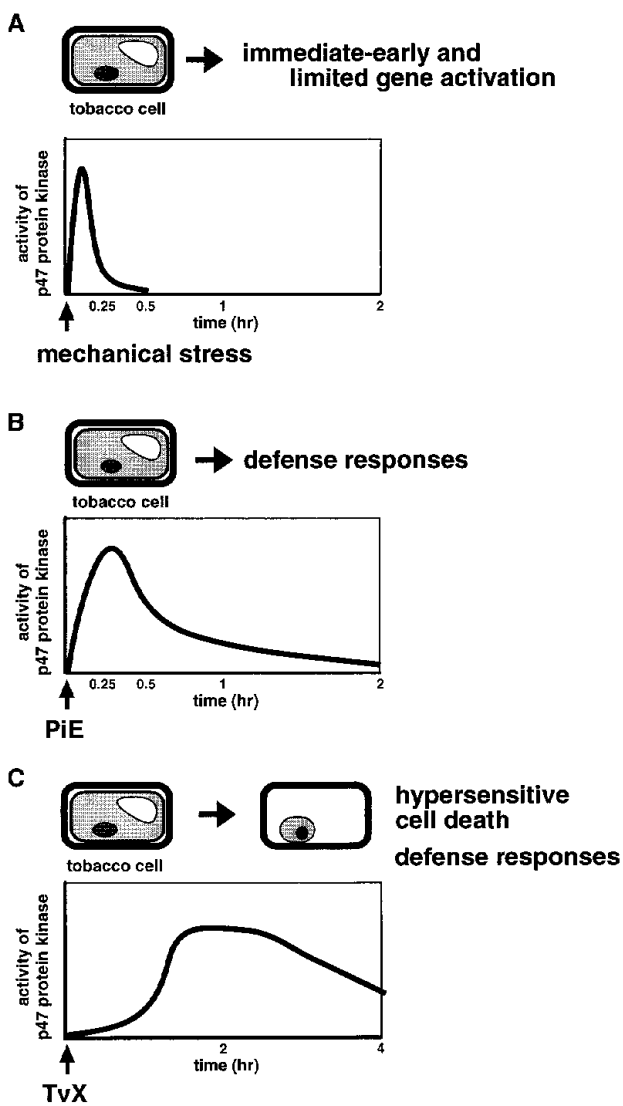

Fig. 2 Schematic representation of timing and duration of activation of p47 protein kinase and subsequent responses in tobacco XD6S cells in response to different extracellular stimuli.

transient activation of the p47 protein kinase might be sufficient for initiation of defense responses such as the oxidative burst and expression of defense genes (**Fig. 2B**). Furthermore, it is plausible that the elicitor-induced slow and prolonged activation of the p47 protein kinase might be a prerequisite for hypersensitive cell death (**Fig. 2C**).

A similar paradigm has been suggested for the mammalian MAPK pathway. Several studies have revealed the importance of the duration of activation of c-Jun N-terminal kinase (JNK)/stress-activated kinase (SAPK) and/or p38 MAPK in determination of cell fate (Xia *et al.*, 1995; Chen *et al.*, 1996a and b; Goillot *et al.*, 1997). In Jurkat T-cells, T-cell activation signal induced the rapid and transient activation of JNK and the proliferation of T-cells, by contrast a lethal dose of γ radiation or UV-C induced the delayed and persistent activation of JNK and apoptotic cell death (Chen *et al.*, 1996a and b). Thus, the p47 protein kinase in tobacco cells appears to be functionally similar to SAPK/JNK and/or p38 MAPK in mammalian cells and the p47 protein kinase may play a role as a component of the elicitor signal transduction (Suzuki and Shinshi, 1996).

In contrast to TvX-induced slow and prolonged activation of p47 protein kinase (Suzuki *et al.*, 1999), the immediate and transient nature of the activation that occurs in response to fungal elicitors appears to be a common feature of various myelin basic protein kinases including MAPKs (Suzuki and Shinshi, 1995; Ádám *et al.*, 1997; Ligterink *et al.*, 1997; Zhang *et al.*, 1998). Both synthesis of protein de novo and protein phosphatase activity might be required for the attenuation of p47 kinase activity and other MAPKs (Suzuki and Shinshi, 1995, Meskiene *et al.*, 1998). The process, which induces the delayed and prolonged activation of the p47 protein kinase by TvX, could involve either prolonged activation of upstream kinases or inhibition and/or down-regulation of specific protein phosphatases (**Fig. 3**). The observation that calyculin A induced the prolonged activation of p47 protein kinase (Suzuki and Shinshi, 1995; Suzuki *et al.*, 1999) suggests possible posttranslational negative modulation of the constitutive activity of a p47 protein kinase and its upstream kinases via dephosphorylation-mediated down-regulation.

At 1 μM, staurosporine enhanced TvX-induced cell death and the activation of p47 protein kinase (Suzuki *et al.*, 1999), whereas it inhibited the TvX-induced defense responses (Suzuki *et al.*, 1999), the PiE-induced activation of the p47 protein kinase (Suzuki and Shinshi, 1995) and the expression of defense genes (Suzuki *et al.*, 1995). By contrast, the

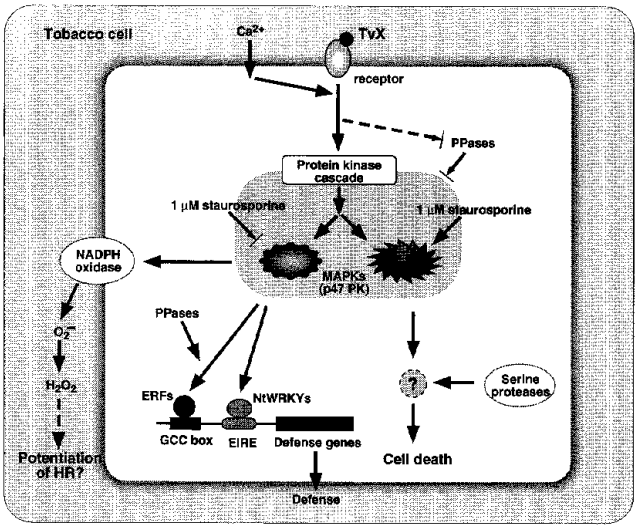

Fig. 3 Proposed model for TvX-activated signaling pathway that leads to defense responses and hypersensitive cell death in tobacco XD6S cells. p47 PK, p47 protein kinase. PPases, protein phosphatases.

staurosporine at 10 μM prevented the TvX-induced activation of the p47 protein kinase and cell death as well as the defense responses (Suzuki *et al.*, 1999). Therefore, we postulated that TvX might activate distinct forms of p47 protein kinase, which have varying sensitivities to staurosporine and which are involved in the induction of varying cellular responses (**Fig. 3**).

5. Involvement of Ca^{2+} in elicitor signal transduction.

An influx of Ca^{2+} ions has been implicated in the activation of early defense responses induced by elicitors (Kombrink and Somssich, 1995; Ebel and Mithöfer, 1998). In addition, an influx of Ca^{2+} ions across the plasma membrane might be involved in the bacterial induction of hypersensitive cell death in plants (Atkinson *et al.*, 1990; Levine *et al.*, 1996) and such an influx of Ca^{2+} ions has often been implicated in the induction of apoptosis in animal cells (Martin JS *et al.*, 1994). In human B lymphocytes, apoptosis and sustained activation of SAPK and p38 MAPK, which were induced by cross-linking of membrane IgM, were inhibited by a calcium-channel blocker (Graves *et al.*, 1996). We have also shown that a calcium-channel blocker prevented not only sustained activation of the p47 protein kinase but also induction of hypersensitive cell death in XD6S cells treated with TvX (Yano *et al.*, 1998; Suzuki *et al.*, 1999). Our results have shown that the entry of extracellular Ca^{2+} ions might be required for the induction of a diverse array of

early events in tobacco cells in response to PiE and TvX (Suzuki *et al.*, 1995; Suzuki and Shinshi, 1995; Fukuda, 1996; Yano *et al.*, 1998; Suzuki et al, 1999). Therefore, it is likely that Ca^{2+} ions might function at immediate downstream of the initial elicitor-receptor interaction **(Fig. 3)**.

6. Involvement of reactive oxygens and proteases in elicitor signal transduction.

The rapid production of reactive oxygen intermediates (ROIs), which include superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), by plant cells that have been challenged by pathogens is one of the most striking events during the early phase of the HR. The oxidative burst has been shown to be induced via a protein kinase cascade. ROIs have been implicated in multiple phenomena that occur during plant defense responses (for review see Doke *et al.*, 1996; Low and Merida, 1996; Wojtaszek, 1997). H_2O_2 has been postulated to be a mediator of defense gene expression and cell death in soybean culture (Levine *et al.*, 1994). Recent report suggests that ROI may be also rapidly induced at distal uninfected leaves following a local oxidative burst in infected leaves and function as a second messenger for systemic acquired resistance (Alvarez *et al.*, 1998). Superoxide is also a key regulator of induction of the spreading cell death associated with the *lsd1* mutant of Arabidopsis (Jabs *et al.*, 1996). Furthermore, nitric oxide (NO) has recently been identified as a second messenger during HR (Delledonne *et al.*, 1998; Durner *et al.*, 1998).

However, it has been also reported that a normal level of ROIs production by the oxidative burst is not sufficient to trigger cell death (Glazener *et al.*, 1996; Jabs *et al.*, 1997). PiE did not induce cell death in XD6S cells, even though the level of ROIs from the oxidative burst after treatment was similar to that observed after cells had been treated with TvX (Suzuki, K and Shinshi, H., unpublished result). This result is consistent with the results reported by Glazener *et al.* (1996) and Jabs *et al.* (1997). In addition, co-treatment of XD6S cells with xanthine and xanthine oxidase, which were used as an extracellular superoxide-generating system (Jabs *et al.*, 1996), did not induce cell death (Yano *et al.*, 1999). The degradation of H_2O_2 by catalase, the scavenging of ROIs by *N*-acetyl-L-cysteine and the prevention of production of O_2^- by inhibition of NADPH oxidase by diphenyleneiodonium, which completely inhibited the TvX-induced accumulation of H_2O_2 in the culture medium, did not affect the TvX-induced cell death (Yano *et al.*, 1999). We have also found that the TvX induces activation of p47 protein kinase even in the presence of such inhibitors (A. Yano, K. Suzuki, and H. Shinshi, unpublished results). These results suggest that accumulation of ROIs is not necessary for the induction of TvX-mediated activation of p47 protein kinase and cell death and it is possible that TvX induces cell death in tobacco cells via a ROS-independent signaling pathway which is distinct from the ROI-mediated pathway (Yano *et al.*, 1999). However, ROI (and probably NO) produced by the TvX-induced oxidative burst might act to potentiate defense responses and hypersensitive cell death, since a variety of experiments strongly support a model whereby ROI and NO potentiate the overall HR in plant (for review see Richberg *et al.*, 1998).

In animal cells, members of a family of cysteine proteases, designated caspases, have been shown to play important roles in the regulation of the development of programmed cell death (pcd) (Nicholson and Thornberry, 1997). Although no homologs of caspases have been identified in plant cells, it was shown that possible involvement of activity of cysteine protease and/or caspase-like protease but not of serine protease in induction of HR (del Pozo and Lam, 1998; Solomon *et al.*, 1999). On the contrary, a serine protease might be involved in the early signaling of hypersensitive cell death because inhibitors of serine proteases, such as leupeptin, 3,4-dichloroisocoumarin, and 4-(2-aminoethyl)-benzenesulfonyl-flonyl-flouride (AEBSF), but not of caspases prevented the TvX-induced cell death and the inhibitory effect was observed during only early phase of the cell death (Yano *et al.*, 1999).

7. Regulatory mechanism of elicitor-inducible defense gene expression

To elucidate the regulatory mechanism of expression of defense genes, we are investigating the *cis*- and *trans*-acting elements that are involved in transcription of genes for class I basic chitinases and class I basic β-1,3-glucanase responsive to fungal elicitors. In tobacco XD6S cells, it has been shown that PiE and/or TvX activates transcription of *CHN48*, *CHN50* and *GLN2* (Fukuda and Shinshi, 1994; Suzuki *et al.*, 1995; Yamamoto, S., Suzuki, K., and Shinshi, H., in preparation). Deletion analysis of the promoter of *CHN50* in transgenic XD6S cells showed that the region of the gene between positions -788 and -345 from the site of initiation of transcription is sufficient for the PiE-induced activation of transcription (Fukuda and Shinshi,

1994). This region contains a putative elicitor-responsive element (ElRE, Fukuda and Shinshi, 1994) and an ethylene-responsive element (ERE), namely, GCC box (Shinshi *et al.*, 1995). The ElRE contains a direct repeat of GTCA sequence. The sequence, (T)TGAC(C) or (G)GTCA(A), is also known as conserved motif of W boxes, which has been shown to be functional elicitor-responsive element in parsley *PR1* genes (Rushton *et al.*, 1996) and may be a general feature of promoters of a large subset of elicitor-inducible defense genes (Rushton and Somssich, 1998). The presence of elicitor-inducible and sequence specific DNA binding activity that interacts with ElRE in nuclear extracts from elicitor-treated tobacco cells was demonstrated (Fukuda and Shinshi, 1994). In addition, the elicitor-inducible activity of the binding to ElRE was reduced in nuclear extracts prepared from the cells that had been treated with cycloheximide and staurosporine (Fukuda, 1997). Therefore, it is tempting to speculate that elicitor signal may be transmitted to the DNA binding protein and activate the transcription of the chitinase genes via protein kinase cascade including the p47 protein kinase (**Fig. 3**).

Three parsley W box-binding proteins, namely, WRKY1, 2, and 3, were identified (Rushton *et al.*, 1996). The mRNAs corresponding to WRKY1 and WRKY3 are rapidly and transiently increased parsley cells in response to fungal elicitor treatment (Rushton *et al.*, 1996) and WRKY1 may function as a transcriptional activator mediating fungal elicitor-induced gene expression via W box elements (Eulgem *et al.*, 1999). Recently, we identified four tobacco genes encoding proteins that are homologous to WRKY proteins, namely, NtWRKY1, 2, 3, and 4 (Yamamoto, S., Suzuki, K., Yano, A., and Shinshi, H., in preparation). These proteins are conceivable candidates for transcriptional regulators to modulate elicitor-inducible transcription of genes via (T)TGAC(C) motif in functionally defined ElRE (**Fig. 3**).

A GCC box is found in the promoter regions of a large number of defense genes (Ohme-Takagi and Shinshi., 1990; Zhou *et al.*, 1997) and has been shown to function as an ethylene-responsive element (Shinshi *et al.*, 1995; Ohme-Takagi and Shinshi, 1995; Suzuki *et al.*, 1997). Specific binding proteins to the GCC box have been identified and called as ERFs, formerly designated as EREBPs (Ohme-Takagi and Shinshi, 1995). We also found that level of mRNAs for all ERFs were increased by ethylene and wounding (Ohme-Takagi and Shinshi, 1995; Suzuki *et al.*, 1997). Recently, we found that TvX induced gradual increase of ERF2 mRNA in contrast to transient increase of mRNAs for ERF3 and ERF4 and the activation of transcription of gene via the GCC box independently of ethylene production in tobacco XD6S cells (**Fig. 3**, Yamamoto, S., Suzuki, K., and Shinshi, H., submitted). In addition, it was suggested that both protein phosphorylation and dephosphorylation might be required for the TvX-induced expression of gene for ERF2 and for the TvX-activated GCC box-mediated transcription of gene (**Fig. 3**).

8. Perspectives

As described here, an availability of the model system should facilitate the biochemical and molecular biological analysis of the elicitor-inducible processes that lead to HR, and the system should also provide a tool for the isolation and identification of the specific components that are responsible for perception of elicitor, elicitor signal transduction, transcriptional regulation of defense genes, hypersensitive cell death and also survival. For example, identification of the AEBSF-sensitive serine protease and cloning of the corresponding gene will advance our understanding of the role of this protease in not only hypersensitive cell death but also programmed cell death in plant. The isolation of binding proteins for TvX will provide important information on the molecular identification of receptor protein for TvX, since the xylanase activity of TvX is probably not required for the elicitation process (Sharon *et al.*, 1993), and the high-affinity binding site for TvX has been identified on plasma membrane in tobacco cells (Hanania and Avni, 1997). The molecular identification of the p47 protein kinase will help us to elucidate the role of this kinase in the elicitor signal transduction and the possible relationships between this kinase and known tobacco MAPKs, which seem to function as signal mediators responsive to various stimuli (Wilson *et al.*, 1993; Wilson *et al.*, 1995; Seo *et al.*, 1995; Zhang and Klessig, 1997). The future analyses of function of the components identified in this system will also contribute to our understanding of the common mechanism of signal transduction in response to environmental stimuli in plant.

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Transgenic Improvement of Photosynthetic Property in Tobacco Using El2 Ω Promoter System to Express Higher Level of Mouse Carbonic Anhydrase Activity in Cytoplasm

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Abstract

Genetic challenge to express higher carbonic anhydrase (CA) activity in the cytoplasm of tobacco mesophyll cells was carried out by an improved promoter (El2 Ω) with a cDNA of partially modified mouse CA. In leaves of T1 progenies of transgenic tobacco (ffCA plant), the expressed mouse CA peptide was confirmed with 1.5 - 3.2 times higher than the transgenic tobacco having foreign mouse CA controlled by simple CaMV 35S promoter (fCA plant). The ffCA plants manifested higher photosynthetic carbon assimilation (PCA) rate under ambient CO_2 condition than wild type plants. The chlorophyll fluorescence measurement analysis revealed that the ffCA plant's improved PCA rate is related to the reduction of mesophyll CO_2 transfer resistance (Rr) with little change in the stomatal resistance (Rs) or mesophyll CO_2 fixation resistance (Rx). Moreover, ffCA plants showed a higher growth rate under high light condition.

Abbreviations

CA: carbonic anhydrase, CaMV: cauliflower mosaic virus, ffCA plants: the transgenic tobacco having foreign mouse CA controlled by El2 Ω promoter, fCA plants: the transgenic tobacco having foreign mouse CA controlled by simple CaMV 35S promoter, PCA: photosynthetic carbon assimilation, Rr: mesophyll CO_2 transfer resistance, Rs: stomatal resistance, Rx: mesophyll CO_2 fixation resistance, WA unit: Wilbur-Anderson CA activity unit

1. Introduction

Plant biotechnology in photosynthetic improvement is based on finding the limiting steps in the metabolic pathway in the plant's cellular physiology. Carbonic anhydrase [EC4.2.1.1, CA], which catalyzes the conversion reaction between CO_2 and HCO_3^- and also promotes the inorganic carbon diffusion through lipid membranes (Broun *et al.*, 1970), is highly related to the flow of carbon in the leaf photosynthesis. Although there have been some reports suggesting the significant existence of extra-chloroplastic CA isozymes in several C3 species (Utsunomiya and Muto, 1993; Rumeau, 1996), most CA activity characteristically localizes in the chloroplastic stroma and not in its envelope (Tsuzuki *et al.*, 1985). In the previous report (Jinushi *et al.*, 1998), based on a very low level of cytoplasmic intrinsic CA activity we generated transgenic tobacco (fCA plants) which expresses foreign mouse CA in the mesophyll cytoplasm controlled by a conventional cauliflower mosaic virus 35S (CaMV 35S) promoter. The fCA plants which expressed foreign CA at high levels showed a significant and consistent enhancing tendency in the photosynthetic carbon assimilation (PCA) rate under lower CO_2 conditions. However, the strain that expressed foreign CA at a lower level did not show such an enhancement. These facts lead to hypothesis that the introduction of higher foreign CA activity into the cytoplasm would enhance its PCA activity to a greater extent.

Among the unique techniques available two reports were suggestive; (1) Kay *et al.* (1987) reported that the duplication of CaMV35S promoter sequences enhanced foreign gene expressions. (2)

Mitsuhara *et al.* (1996) constructed a new CaMV35S promoter (El2 Ω) containing two extra-enhancer sequences; one is El2 sequence which is in the upstream region of CaMV35S promoter, and the other is Ω sequence which is the 5'-untranslated region of tobacco mosaic virus genomic RNA. They were observed to have more than 10 times higher expression of the marker gene using El2 Ω promoter compared with a single CaMV35S promoter in the transgenic tobacco.

In this paper we describe a study using El2 Ω promoter in the production of unique transgenic tobacco plants (ffCA), into which have been introduced a partially modified mouse CA cDNA and which expressed mouse CA peptide in the mesophyll cytoplasm. We measured those mesophyll resistances for CO_2 transfer (Rr) in photosynthesis with chlorophyll fluorescence methods in order to elucidate the detailed mechanisms of introduced foreign CA on PCA.

2. Materials and Methods

2.1 Construction of gene cassette with improved promoter and transformation

The vector plasmid pBffCA010 was constructed by replacing the sequence of β-glucuronidase gene and nos-terminator of the pBE2113-GUS (Mitsuhara *et al.*, 1996) with the sequence of the partially modified mouse CA cDNA and nos-terminator in the pBfCA010 (Jinushi *et al.*, 1998) of 861 bp at *Bam*HI-*Eco*RI site (**Fig.1**). It was introduced into leaf discs of tobacco (*Nicotiana tabacum* line SR-1) according to the same method as previously reported (Jinushi *et al.*, 1998; Horsch *et al.*, 1985). Transgenic tobacco plants were screened by antibiotic Km resistance and the expression level of foreign CA peptide using anti-serum against bovine CA type II which could discriminate it from plant intrinsic CA peptides.

2.2 Plant Materials

T1 seeds of the transgenic tobacco plants, the strain ffCA-3, which showed an extremely high level of foreign CA expression, were sown on sand. The seedlings were grown in the growth cabinet where the temperature was maintained at 25℃/20℃ (day/night) with a 12 hour day length. The light intensity was kept constant at 1000 μmol · m^{-2} · s^{-1} during day time. Two sets of the plants were prepared; one for the measurement of leaf intrinsic CA and foreign CA activity, and the other for the measurement of leaf PCA properties.

2.3 Determination of CA Activity

In the leaves of line SR-1, which originated from the plastid genome mutation (Svab and Maliga, 1991), there were extremely low levels of endogenous CA activity compared to those of c.v. Xanthi, which we had previously tested to generate fCA transgenic plants (Jinushi *et al.*, 1998). Fifteen cm^2 of the leaves was ground with the mortar and pestle for 2 min on ice with 4 % of polyvinyl polypyrrolidon, sea-sand and 1.4 m*l* of the extraction buffer (pH 8.3) containing 50 mM sodium barbital, 1 mM Na_2-EDTA, 2 % glycine, 2 % polyvinyl pyrrolidon 1 % BSA, 10 % glycerol and 0.2 mM phenylmethanesulfonylfluoride. After centrifugation at 4 ℃ and 15,000 x g for 5 min, an aliquot of the supernatant was used for the measurement of CA activity (Tsuzuki *et al.*, 1985). The foreign CA activity in the leaf extract was estimated by differential measurement in the presence and absence of 10^{-7} M acetazolamide which inhibited the animal CA type II by 99 % without any effect on leaf endogenous CA (Jinushi *et al.*, 1998).

2.4 Gas Exchange Measurement

The PCA rates and fluorescence signals were simultaneously determined in the youngest fully

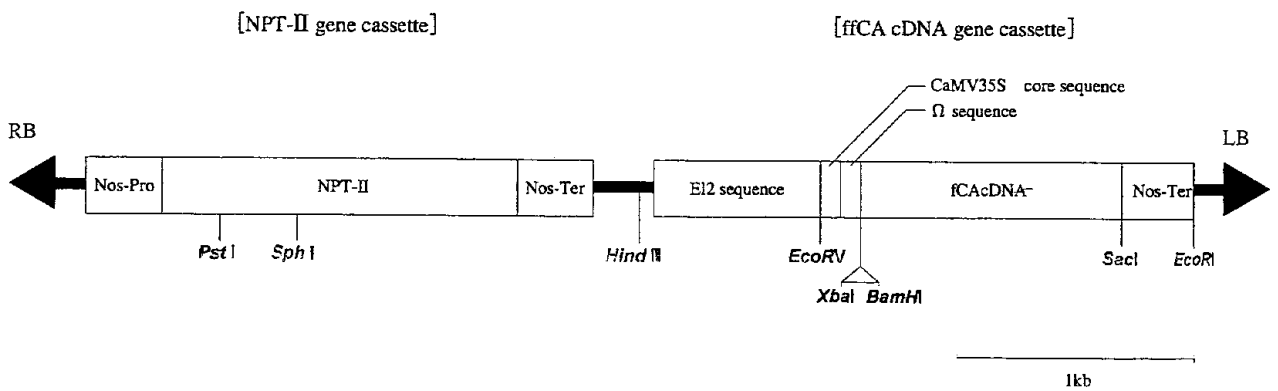

Fig.1 The vector construction of pBffCA010; the ffCA cDNA gene cassette which was designed to express the partially modified mouse CA type II protein with total size of 252 amino acids at the left border and the NPT-II gene cassette for the screening of transgenic plants at the right border.

expanded leaves. In order to confirm the unique effects of cytoplasmic foreign CA activity specifically, sample plants with similar leaf conditions were selected by leaf chlorophyll content measured by a non-destructive method. The measuring system for the PCA rate was the same as reported in the previous paper (Jinushi *et al.*, 1998; Nobel *et al.*, 1975). The ambient CO_2 concentration was sequentially changed in the order of 350, 200, 100 and 80 ppm. The light intensity, and the leaf temperature were maintained constant at 1000 μ E · m^{-2} · s^{-1} and 26 ± 1 ℃, respectively. In some measurements, PCA was also determined under 350 ppm of CO_2 at 500 μ E · m^{-2} · s^{-1} of the light intensity.

2.5 Chlorophyll Fluorescence Analysis

To estimate Rr, chlorophyll fluorescence parameters were measured with a PAM 101 modulated fluorometer (Waltz) (Genty *et al.*, 1989; Harley *et al.*, 1992; Schreiber, 1986). To measure the fluorescence signals simultaneously with PCA determination, a polyfurcated optic fiber was supported at angle of 45° to the leaf chamber to avoid the shading of the leaf light illuminating.

The measurement of Rr value is known to be rather stable under lower Ci conditions (Loreto *et al.*, 1992), thus, we compared the Rr values at Ci = 100 ppm. The calculated Rr value of Wild types of tobacco was nearly the same level as those reported in other papers which measured using the isotopic carbon method (Evans *et al.*, 1986; Evans *et al.*, 1994; von Caemmerer and Evans, 1991).

2.6 Western Blot Analysis

The RubisCO and expressed foreign CA levels in the leaves were measured by SDS-PAGE and Western blot analysis as described in the previous paper (Jinushi *et al.*, 1998).

2.7 Growth Analysis

Using non-destructive leaf area measurement with a video camera and 2-dimension pattern analysis program (ATTO, Japan), we selected transgenic and wild type seedlings of similar size at 5 weeks after sowing. They were cultured for 17 or 30 days under natural light or 50 % shaded conditions in the green house. The temperature was maintained at 25 ℃ /20 ℃ (day/night). Day time light intensity was at least 1000 μE · m^{-2} · s^{-1} and the CO_2 concentration was around 390 ppm.

3. Results and Discussion

The transgenic plants which showed a high level of foreign CA protein were confirmed by Western blot analysis of the leaf extracts of individuals of 4 strains (ffCA-1, -3, -6 and -7) among the 14 seedlings recovered from the screening antibiotic Km medium. By the PCR Southern and Northern analyses, it was confirmed that the mouse ffCA cDNA gene cassette existed in the chromosomal genome and was transcribed to messenger RNA in these 4 strains. However, two plantlets of these strains showed a strange appearance; the strain ffCA-6 was dwarf with slender leaves, probably due to somaclonal variation and the strain ffCA-7 was sterile. Therefore, T1 seeds were obtained only from two remaining strains (ffCA-1 and ffCA-3). The segregation ratios of foreign CA expressing and non-expressing phenotypes in T1 plants were 27:0 (ffCA-1) and 33:4 (ffCA-3), which indicated that at least two copies of mouse CA cDNA gene cassette were introduced into the chromosomal DNA in these transgenic plants. **Fig. 2** shows the Western blot analysis of leaf extracts in ffCA plants (ffCA-3) under the control of El2 Ω promoter and of fCA plants under the control of single CaMV35S promoter at 6 weeks after sowing. The expression level of foreign CA peptide in ffCA-3 was calculated with the 2-dimentional-density pattern analysis program to be 3.2 times higher than the highest expression among the 23 fCA plants (High CA Plant). This indicated that the El2 Ω promoter could enhance the mouse CA cDNA gene expression just like GUS gene reported before (Mitsuhara *et al.*, 1996). On the other hand, ffCA-1 showed a lower

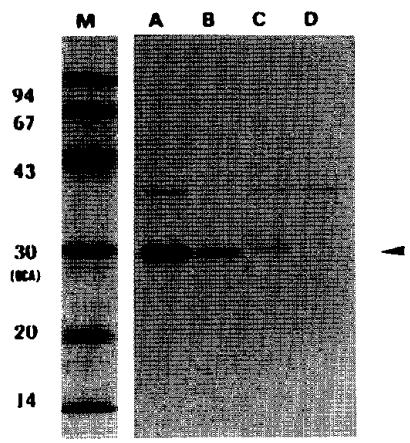

Fig. 2 Comparison of mouse CA expression level between transgenic plants containing different promoters. Lane M shows the molecular weight markers (Pharmacia) with CBB staining. The lanes A, B, C, and D, show the immuno-detection of the leaf extract in the ffCA transgenic plant (the strain ffCA-3), in the High CA Plant and Low CA Plant of the fCA transgenic plant and in Wild Type, respectively. The arrow indicates the immuno-detection band of about 29 KD.

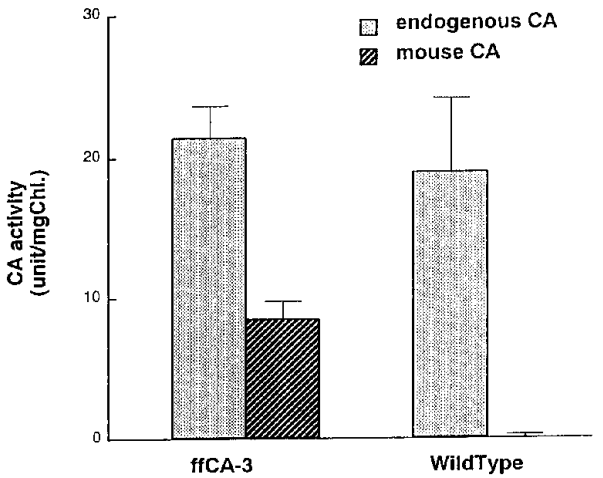

Fig. 3 Partially modified mouse CA activity in the leaf of ffCA transgenic plant (the strain ffCA-3).

expression level of foreign CA compared to ffCA-3 (data not shown). From the differential CA activity measurements with and without acetazolamide (Jinushi *et al.*, 1998), the expression levels of foreign CA activity were estimated as 8.39 ± 1.41 and −0.01 ± 0.27 WA unit · mg Chlorophyll^{-1} in the leaves of the strain ffCA-3 and wild type, respectively. Thus, foreign CA activity occupied as high as 39.2 % of total leaf CA activity in ffCA-3 (**Fig.3**). Specific activity of expressed foreign CA in ffCA plants were, nearly the same level (800 ± 133 WA unit / mg CA peptide) as those of fCA plants in the previous experiment with c.v. Xanthi (841 ± 58 and 851 ± 163, for High CA phenotype and Low CA phenotype, respectively) (Jinushi *et al.*, 1998).

At 8 weeks after sowing, the average expression level of foreign CA was 425.1 ± 15.0 ng · cm^{-2} in the T2 plant of ffCA-3 (**Table 1**). While leaf RubisCO contents in ffCA plants showed no difference from that in the wild type. The PCA rates of ffCA-3 were significantly higher than those of the wild type even at the ambient air condition (Ci = 250ppm) where little improvement could be seen in the fCA previously reported (Jinushi *et al.*, 1998) (**Table 2**). At 250 ppm of Ci, the PCA rate of the transgenic plant was 14.1 ± 0.1 μmol CO_2· m^{-2}· s^{-1}, which was 2.9 % higher than that of the wild type. Moreover, at 100 ppm of Ci, the transgenic plants showed 6.7 % higher PCA rate than that of the wild type. However, compared to fCA plants the percentages of the improvement of PCA rate at lower CO_2 concentration did not increase drastically in ffCA plants. This might indicate the existence of a certain saturation level of cytoplasmic foreign CA activity for the improvement of PCA rate in tobacco plants. Regarding this point, it was reported that Rr might not be a major limiting step in the C3 PCA process (Sasaki *et al.*, 1996). Our data showed little effects of foreign CA on the PCA rate at such a low light

Table 1. The levels of mouse CA, RubisCO and chlorophyll in the leaves of ffCA (SR − I) and fCA (Xanthi) transgenic tobacco plants.

	The leaf content of		
	mouse CA	Rubis CO	chlorophyll
	ng cm^{-2}	μ g cm^{-2}	μ g cm^{-2}
ffCA plants (n=4)	425.1 ± 15.0	201.9 ± 7.7	40.5 ± 1.0
Wild type (n=5)	---	207.7 ± 5.5	41.3 ± 1.1
Reference data from the previous report with Xanthi			
fCA plants (n=9)	277.0 ± 8.1	203.0 ± 4.8	40.4 ± 0.6
Wild type (n=8)	---	207.8 ± 4.1	41.7 ± 0.8

Table 2. The PCA rate in ffCA transgenic plants under high light condition of 1000 μ molE^{-2} S^{-1}.

	PCA rate at CO_2 concentrations of			
	Ci300ppm	Ci250ppm	Ci150ppm	Ci100ppm
	μ mol CO_2 m^{-2} s^{-1}			
ffCA plants (n=4)	16.9 ± 0.2*	14.1 ± 0.1**	8.21 ± 0.05***	4.48 ± 0.05**
Wild type (n=5)	16.5 ± 0.1	13.7 ± 0.1	7.78 ± 0.10	4.20 ± 0.08
Enhancement (ffCA/WT) x100	2.4%	2.9%	5.5%	6.7%

*, ** and ***: 5, 2.5 and 1% significance, respectively.

intensity as 500 μ E · m^{-2} · s^{-1} and a higher CO_2 concentration such as 300 ppm, namely, 6.65 ± 0.16 and 6.79 ± 0.21 μmol CO_2 · m^{-2} · s^{-1}, ffCA plants (n=4) and wild type (n=5), respectively. It can be concluded that the optimal manifestation of the cytoplasmic CA introduction effect on C3 photosynthesis is only observable under light saturated and CO_2 supply limiting conditions.

We also conducted the simultaneous measurement of chlorophyll fluorescence parameters. No significant differences in the calculated quantum yield of PS Ⅱ (Φ Ⅱ) was observed between ffCA transgenic plants and the wild type (data not shown). However, the Rr of ffCA plants was significantly lower than that of the wild type. On the other hand, we could detect no differences in stomatal resistance (Rs) and mesophyll resistance for CO_2 fixation (Rx) between ffCA and wild type **(Table 3)**. Therefore, these results indicated that the improvement of PCA rate under CO_2 limiting conditions occurrs with the introduction of foreign CA activity in mesophyll cytoplasm through the reduction of Rr. The failure to detect any significant improvement in PCA rate under lower light conditions where Rr was not a limiting factor in PCA (von Caemmerer and Farquhar, 1981) would support this hypothesis.

As to the growth observation under the natural light condition, the high - ffCA transgenic plants grew faster than the wild type plants. The seedlings of strain ffCA - 3 showed 10 % larger leaf area and 7 % heavier total dry weight than wild type plants at 7 weeks after sowing **(Table 4)**, although there was no significant change in the low - ffCA transgenic plant, ffCA - 1. In the similar experiment under low light condition with 50 % shading of the natural light intensity, ffCA - 3 did not show any growth

Table 3. Total resistance (Rt), stomatal resistance (Rs), mesophyll resistance for CO_2 transfer(Rr) and mesophyll resistance for CO_2 fization (Rx) in ffCA transgenic plants under the condition of 1000 μ molEm^{-2} s^{-1} of light intensity and Ci=250ppm.

	Rt	Rs	Rr	Rx
	molm^{-2} s^{-1}			
ffCA plants (n=4)	20.9 ± 0.2**	3.18 ± 0.10	6.20 ± 0.18**	11.5 ± 0.2
Wild types (n=5)	21.7 ± 0.2	3.44 ± 0.19	6.81 ± 0.17	11.4 ± 0.1

**: 2.5% significance.

Table 4. Leaf area, total dry weight, mouse CA and chlorophyll content in the ffCA transgenic plants under natural light condition.

	Total Leaf Area		Total Dry Weight	Leaf mouse CA	Leaf Chl.
	cm^2		g	ng · cm^{-2}	μg · cm^{-2}
	5weeks	7weeks	7weeks	7weeks	7weeks
ffCA-3 (n=12)	17.1 ± 0.5	532.4 ± 15.0**	1.69 ± 0.05*	235.4 ± 23.6	33.2 ± 0.4*
ffCA-1 (n=7)	17.0 ± 0.4	486.9 ± 18.8	1.55 ± 0.07	80.3 ± 14.3	32.7 ± 0.6
Wild type (n=10)	17.1 ± 0.4	483.9 ± 15.1	1.58 ± 0.05	–	31.7 ± 0.8

* and **: 5 and 2.5% significance, respectively.

Lable 5. Leaf area, total dry weight, mouse CA and chlorohpyll content in the leaf of ffCA trnasgenic plants under 50% shading natural light condition.

	Total Leaf Area		Total Dry Weight	Leaf mouse CA	Leaf Chl.
	cm^2		g	ng · cm^{-2}	μg · cm^{-2}
	5weeks	9weeks	9weeks	9weeks	9weeks
ffCA-3 (n=4)	10.0 ± 0.5	449.6 ± 24.6	0.97 ± 0.08	116.4 ± 9.2	27.6 ± 0.5
Wild type (n=7)	10.3 ± 0.4	502.4 ± 20.8	1.07 ± 0.05	–	27.6 ± 0.4

enhancement (**Table 5**). These findings corresponded well to those of photosynthetic analysis data and suggested the positive effects of introduced cytoplasmic CA on their growth rate under light saturated and CO_2 supply limiting conditions. However, there remain alternative possible indirect reasons induced by the introduction of foreign CA activity in the cytoplasm connected to the observed growth enhancement: 1) Leaf chlorophyll and RubisCO contents increased along with the plant size in the vegetative stage of tobacco seedlings. Hence, the transgenic plants with higher PCA activity might have relatively thick and photosynthetically active leaves at an earlier growing stage compared with the wild type plants (**Table 4**). 2) When transgenic plants grew faster for the reasons above, they might have expanded their leaves faster than wild type plants. Therefore, they might have higher light capturing efficiency at the whole plant level, which brought about further growth enhancement. 3) Under extremely high light conditions, stomatal closure would lead a decrease in the internal CO_2 concentration of the leaf bringing about photosynthetic photoinhibition. Enhancement of leaf carbon utilization efficiency by the introduced cytoplasmic CA activity might prevent mesophyll tissues from accumulating extra-reducing power. Regarding this, the reduction of photoinhibition is one of the major target points of the recent crop breeding challenge in molecular biology.

Price *et al.* (1994) carried out suppression experiments of endogenous CA activity by means of anti-sense mRNA technology and reported that the drastic reduction of tobacco leaf endogenous CA activity to 1 or 2 % of wild type made little effect on its PCA rate. Thus, it was considered that there should be too much CA activity in the mesophyll cells of C3 plants and any over-expression of CA activity in the mesophyll cells (in the chloroplasts) might not contribute to improvement of its PCA rate (Majeau *et al.*, 1994). However, the results of our previous and present paper suggested that the level of CA activity in choloroplasts in C3 tobacco appears to be saturated in nature but the specific introduction of CA activity into the cytoplasmic area of mesophyll cells would improve PCA activity through reduction of Rr even with a small increase range of the introduced CA activity.

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