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BRIEF COMMUNICATION

Impaired plant growth and development caused by human immunodeficiency virus type 1 Tat

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Abstract Previous attempts to express the human immunodeficiency virus 1 (HIV-1) Tat (trans-activator of transcription) protein in plants resulted in a number of physiological abnormalities, such as stunted growth and absence of seed formation, that could not be explained. In the study reported here, we expressed Tat in tomato and observed phenotypic abnormalities, including stunted growth, absence of root formation, chlorosis, and plant death, as a result of reduced cytokinin levels. These reduced levels were ascribed to a differentially expressed CKO35 in Tat-bombarded tomato. Of the two CKO isoforms that are naturally expressed in tomato, CKO43 and CKO37, only the expression of CKO37 was affected by Tat. Our analysis of the Tat confirmed that the Arg-rich and RGD motifs of Tat have functional relevance in tomato and that independent mutations at these motifs caused inhibition of the differentially expressed CKO isoform and the extracellular secretion of the Tat protein, respectively, in our Tat-bombarded tomato samples.

Keywords Cytokinin · Cytokinin oxidase · HIV-1 Tat · Tomato · Transient expression

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Introduction

Plants have a great potential as "green factories" for the production of medicines (Zahn et al. 2008), including vaccines against human immunodeficiency virus (HIV)-1 (Karasev et al. 2005; Ramirez et al. 2007; Shchelkunov et al. 2006). There are numerous advantages to adopting a plant-made vaccine strategy for managing the acquired immunodeficiency syndrome (AIDS) pandemic, such as the induction of mucosal immunity when vaccines are in edible form, easy storage of plant-made vaccines, no requirement for medical experts, and relatively lower costs (Webster et al. 2005). The Tat (trans-activator of transcription) protein is encoded by HIV-1 and is one of the essential proteins for viral replication (Fisher et al. 1986). Anti-Tat immune responses appear to function in vivo and have often observed in HIV-1-infected individuals prior to the development of full-blown AIDS. Thus, Tat has the potential to be used as a vaccine due to its low variability among HIV-1 subtypes and high conservation among inter- and

intra-patient variants (Addo et al. 2001; Goldstein et al. 2001), allowing efficient CTL induction that would cover a wide variety of HIV-1 clones. However, previous attempts to express the *tat* gene in tomato (Ramirez et al. 2007) and spinach (Karasev et al. 2005) resulted in the development of a number of gross physiological disorders in the transformed plants that could not be explained, demonstrating that there are constraints in the development of Tat-expressing plants.

When Tat is expressed in animal model systems, such as mice, the animals exhibit altered sleep patterns, abnormal circadian rhythms, and fatigue. These changes eventually lead to premature death, but the "cause and effect" is not known (Clark et al. 2005; Kim et al. 2003). Several studies have reported on Tat activity and its effects (Imai et al. 2005; Kanazawa et al. 2000; Muller et al. 1990; Okamoto et al. 2000; Okamoto and Wong-Staal 1986) but, to date, no study has looked at Tat action in plants with the aim of opening up a new way of understanding Tat activity in terms of potential pharmaceutical relevance.

We report here that the tomato plants in our study that transiently expressed HIV-1 Tat had impaired plant growth and development. The phenotypic abnormalities observed included stunted growth, absence of root formation, chlorosis, and eventual plant death. Tat-expressing samples of experimental material contained decreased levels of cytokinin (CK) due to a differentially expressed cytokinin oxidase (CKO). More importantly, we found that the Arg-rich and RGD motifs of Tat allowed Tat to function in the plant system and that by mutating these motifs, we were easily able to avoid the detrimental effects observed in the earlier studies.

Materials and methods

Transient expression of HIV-1 Tat in tomato plantlets

The *tat* gene from the HXB2 strain of HIV-1 was synthesized following a specific codon-usage table based on tomato (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4081). Plasmid pBI121 (Clonetech, San Francisco, CA) was used to express the plant-optimized HIV-1 *tat* gene positioned upstream of a β-glucuronidase (*gus*) gene under the control of 35S promoter and NOS terminator. Tomato variety *Improved Pope* was planted in MS medium (Sigma, St. Louis, MO) and grown for 7–10 days depending on the presence of two distinct leaves which were then used as explant material. Tomato transformation was carried out as previously reported (Evans and Sharp 1983). Briefly, callus induction of the explant material was performed in MS medium containing 1 ppm zeatin (Sigma) to produce tomato calli of varying ages, specifically, 14-, 22-, 33-[termed callus-derived incomplete (CDI)], and 40 (CDC, callus-derived complete stage or plantlet stage)-day-old calli to be used in our study. The vector construct was introduced into the differently aged calli by means of particle-gun transformation. Control plants were bombarded with an empty pBI121 vector. Bombarded tomato calli were grown in MS medium containing 0.5 ppm zeatin, 1 ppm indole-butyric acid and 1 ppm gibberellic acid for 8 weeks. Protein was extracted from all bombarded calli using the P-PER Plant Protein Extraction kit (Thermo Fisher Scientific, Waltham, MA), and the presence of Tat-GUS fusion protein expression was confirmed by western blot assays using anti-Tat (Diatheva, Fano, Italy) and anti-GUS (Invitrogen, Carlsbad, CA) antibodies. Extracts from pBI121-bombarded samples were used as controls. All extracts were also used to check for CK levels using the Phytodetek *t*-ZR Test kit (Agdia; Sigma). The presence of CKO was subsequently confirmed through western blot assays using all of the protein extracts previously obtained together with protein extracts from the shoot apical meristem to serve as a second control. The anti-CKO antibody was a kind gift from Prof. Petr Galuszka (Department of Biochemistry, Palacky University, Czech Republic).

Establishing the extracellular secretion of HIV-1 Tat from tomato plants

Two sets of 14-day-old tomato calli were used to establish Tat extracellular secretion. One set was bombarded with the pBI121-Tat vector and the other set with the empty pBI121 (control). Both bombarded sets were concurrently grown together 3 cm apart following a protocol we had established earlier. We developed a disc-blot immunoassay system to follow Tat secretion. Briefly, we used a grade DE 81

Whatman disc (diameter 2.3 cm) and placed the discs in-between the two bombarded sets overnight on weeks 1, 3, 6, and 8, respectively. The discs were then placed in an Immobilon transfer membrane (Millipore, Billerica, MA) overnight at 4°C, following which the transfer membranes were processed like an ordinary western blot assay (Imai et al. 2005). Tat was detected using the anti-Tat (Diatheva) antibody. The amount of Tat excreted from Tat-bombarded tomato calli was estimated using the Bio-Dot Microfiltration apparatus (Bio-Rad, Hercules, CA) and the Tat protein standard utilizing a serial dilution of recombinant Tat protein (ImmunoDiagnostics, Woburn, MA). Western blot assays were concurrently performed to track Tat expression in the Tat-bombarded and control samples. Plant tissue samples (50 mg) were collected on weeks 1, 3, 6, and 8, and Tat was detected using anti-Tat (Diatheva) antibody.

Detecting the presence of HIV-1 Tat in tomato chloroplasts

Leaf samples from 14-day-old Tat- and pBI121-bombarded tomato plants were collected on weeks 1 and 8. Intact chloroplasts were isolated from the leaf samples using the Chloroplast Isolation kit (Sigma). Chloroplast protein was obtained following the treatment of intact chloroplasts with 10% sodium dodecyl sulfate solution. Western blot assays were performed using chloroplasts isolated from bombarded tomato samples on week 8 and anti-CKO antibody. Western blot assays using chloroplasts from collected from bombarded tomato samples on weeks 1 and 8 and anti-Tat (Diatheva) antibody were also performed. Both western blot assays followed a protocol we established earlier.

Mutagenesis of the Arg-rich and RGD motifs of Tat

Alterations in the Arg-rich (labeled "mArg") and RGD (labeled "mRGD") motifs were made using the GeneTailor Site Directed Mutagenesis System (Invitrogen) and the pBI121-Tat vector as template. Mutagenesis was carried out using the PfuUltra Hotstart DNA Polymerase (Stratagene, La Jolla, CA). The primers used for mRGD were 5′-CCA ACT TCT CAT CTG CTG CTG CTC CAA CTG GAC CAA AG-3′ (forward) and 5′-AGA TGA GAA GTT GGT TGC TTA GAA AGA GA-3′ (reverse), and those used for mARG were 5′-TAT GGA AGA AAG AAG GCT GCT CAA GCT GCT GCT GCT CAT CAA A-3′ (forward) and 5′-CTT CTT TCT TCC ATA AGA AAT TCC AA-3′ (reverse). All constructs were confirmed by sequencing using the ABI PRISM Dye Terminator Cycle Sequencing ready kit (Perkin-Elmer, Foster City, CA) and performed in an Applied Biosystems 313 automated DNA sequencer (ABI, Foster City, CA). Tomato transformation was performed using 14-day-old calli following earlier established protocols. The youngest part of the tomato shoot was used, and protein was extracted. Western blot assays were performed using anti-Tat and anti-CKO antibodies following the protocol we established earlier.

Results and discussion

HIV-1 Tat can affect plants at any developmental stage

To study the effects of the Tat protein expressed in transformed plants, we synthesized a plant-optimized *tat–gus*-fused gene, inserted it into pBI121, transferred the gene construct by particle gun bombardment into tomato calli (at different growth stages/ages) and plantlets, and studied the growth patterns within an 8-week time frame. At week 1, all of the bombarded callus-derived incomplete (CDI) tomato plantlets showed a noticeable growth impairment, such as stunting and the absence of root formation, compared to the control plantlets (Fig. 1a). These observations are similar to those reported by Karasev et al. (2005) in spinach. When young callus-derived complete (CDC) tomato plantlets were bombarded with the construct, growth impairment was not observed during week 1. At week 3, chlorosis (tissue whitening) was evident in the shoot tissues of all Tat-bombarded tomato plantlets (CDI and CDC). Shoot and root development had completely halted in the bombarded CDC plantlets and in CDI plantlets bombarded at 33 days. At week 6, chlorosis was spreading to the entire shoot tissues and by week 8, all developing plant tissues of all bombarded plantlets had turned completely white, with the exception of the CDI plantlets bombarded at 33 days and the CDC

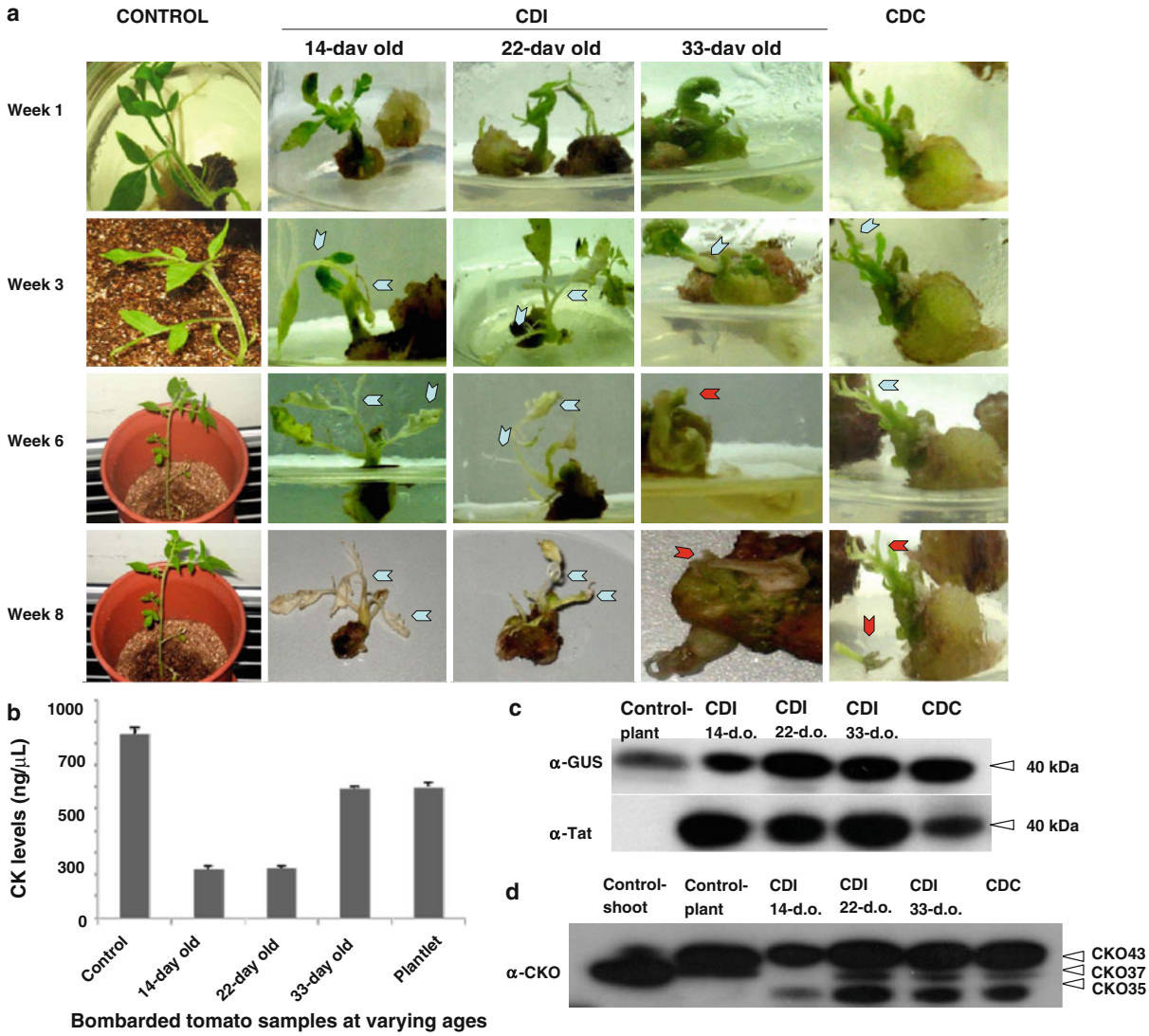


Fig. 1 Reduction of cytokinin and induction of phenotypic abnormalities by transient expression of human immunodeficiency virus (HIV-1) trans-activator of transcription (*Tat*) protein in tomato. **a** Phenotypic abnormalities among 14-, 22-, 33-day-old callus-derived incomplete (*CDI*) and callus-derived complete (*CDC*, 40 days old) tomato plantlets bombarded with Tat and observed within an 8-week time frame. *Control* 22-day-old calli bombarded with an empty pBI121 vector. Presence of chlorosis (*blue chevron*) and necrosis (*red chevron*) are indicated. **b** Abnormal cytokinin (*CK*) levels were measured in Tat-bombarded tomato plantlets. The data represent the means and standard deviations (SD) of three independent measurements. **c** Western blot assays verifying the expression of the Tat–*β*-glucuronidase (*Tat–GUS*) fusion protein using Tat- and GUS-specific antibodies. **d** Detection of tomato cytokinin oxidase (*CKO*) using CKO-specific antibody. CKO43 (43 kDa) and CKO37 (37 kDa) were detected among the control samples, wherein, only CKO37 was present in shoot apical meristem. CKO35 (35 kDa) was detected among Tat-bombarded samples only. *d.o.* Days old

tomato plantlets, both of which exhibited signs of necrosis. Although the root tissues of these plantlets retained some green color, there was a clear failure of root growth at the end of the 8-week period. In addition to exhibiting growth stunting and a failure to form seeds, as reported in the literature (Ramirez et al. 2007), the tomato plantlets were chlorotic and failed to grow roots. These phenotypic abnormalities were observed in all bombarded samples and indicate that Tat can affect the tomato plant at any developmental stage. There have been similar reports of human viral proteins causing mutant phenotypes

when expressed in plants (Kohl et al. 2007; Oey et al. 2009; Santi et al. 2006; Zhou et al. 2008); however, the authors were unable to explain the factors causing these phenotypic abnormalities. Several factors are involved in growth stunting, the inhibition of root growth, the failure to form seeds (Ramirez et al. 2007), and chlorosis. One of these is an alteration and/or disruption of cytokinin biosynthesis (Kakimoto 2003; Sakakibara 2006).

Differentially expressed CKO is produced in the presence of Tat

Cytokinins are a class of plant-specific hormones that play a central role during the cell cycle and influence numerous developmental programs, including root growth and branching, the control of apical dominance in the shoot, chloroplast development, leaf senescence, anthesis, and seed development (Kakimoto 2003; Sakakibara 2006; Werner et al. 2001). Abnormal plant development has been observed to result from experimental alteration of the level of CK (unrelated to Tat levels) (Werner et al. 2001; Yang et al. 2003). The reported abnormalities resemble those observed in our study (Fig. 1a), suggesting that similar deviant CK levels may be found in our Tat-expressing tomato plantlets. We found that the CK levels in bombarded samples were lower than those in our control (Fig. 1b): the levels of CK reduction in the 14-day-old and 22-day-old CDI tomato plantlets were greater that those in the 33-day-old CDI and CDC plantlets. The differences were attributable to the varying developmental stages in which Tat was introduced. Although several factors may affect CK levels (Werner et al. 2001), cytokinin oxidase is one of those regulatory enzymes that affect CK metabolism and hence its levels in the plant (Brugiere et al. 2003; Werner et al. 2001).

Cytokinin oxidase catalyzes the irreversible degradation of all CK in a single enzymatic step by oxidative N^6-side chain cleavage (Brugiere et al. 2003; Schmulling et al. 2003). Changes in CKO activity alter the CK concentration in plant tissues, thereby emphasizing the important role of CKO in controlling local CK levels and contributing to the regulation of CK-dependent processes (Schmulling et al. 2003). We first confirmed the expression of the Tat–GUS fusion protein in all bombarded samples using both Tat- and GUS-specific antibodies (Fig. 1c). CKO from all samples was detected using CKO-specific antibody (Fig 1d). We established that two CKO isoforms exist in tomato, CKO43 (43 kDa) and CKO37 (37 kDa), with the latter found in the shoot apical meristem. It was notable that only the 14-day-old CDI tomato plantlet did not contain CKO37, presumably due to the absence of a shoot apical meristem at this stage. Interestingly, among all of the Tat-bombarded plantlets, CKO35 (35 kDa) was only detected in Tat-bombarded tomato plantlets (bombarded at 40 days, CDC plantlets). Although not proven, given that the Tat protein is a transcriptional activator, it may have specifically promoted the expression of the CKO35 isoform. Yang et al.(2003) reported the existence of alternative spliced forms of CKO, and the CKO35 isoform would seem to be the product of the alternative splicing of CKO37 as a result of Tat presence (Fig. 1b). Our results are the first to demonstrate the unique production of CKO35 production, apparently due to Tat induction. The appearance of the CKO35 isoform coincides in time with the reduction of CK levels in bombarded tissues, suggesting that the presence of CKO35 downregulated CK production.

Arg-rich and RGD motifs of Tat have equivalent functions in the plant system

In terms of the mechanism by which Tat can access the tomato plant system, we speculate that Tat motifs (Albini et al. 1998; Calnan et al. 1991) may have counterparts in plant systems with somewhat similar functions. The RGD motif in Tat acts as an antagonist to integrin receptors in mammalian cells and has been proposed to facilitate the uptake of extracellular Tat (Albini et al. 1998). Plant RGD motifs, which are similar to the mammalian form, are cell-adhesion motifs present in several extracellular matrix proteins (Senchou et al. 2004) and specifically bind to plant-like integrins normally found in the plant cell walls where it has recently been found to be involved in numerous plant processes (Muller et al. 1990). Peptides containing the RGD motif have been found to penetrate and easily pass through the cell wall (Meinhardt et al. 2002). The questions to be answered are whether the transiently expressed Tat–GUS fusion protein can penetrate the plant cell walls of the bombarded tomato calli and if it is extracellularly secreted, i.e., whether the protein can affect

neighboring control tomato plantlets and show similar symptoms.

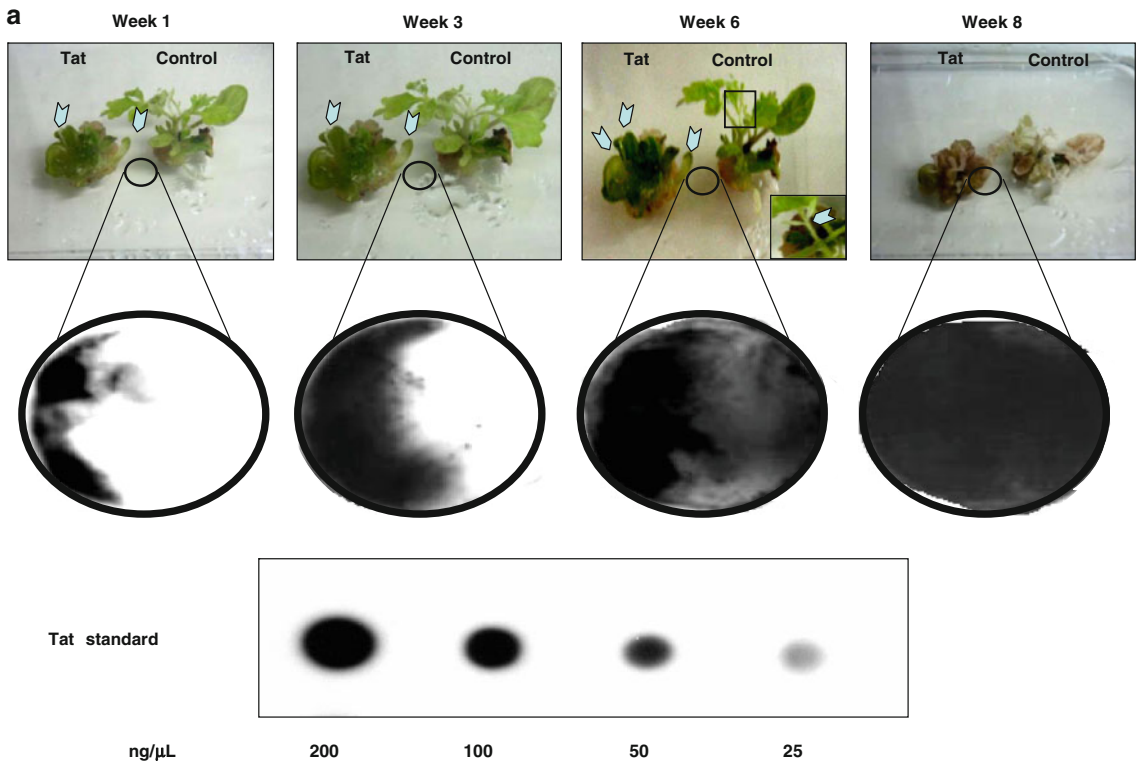
When two sets of 14-day-old tomato calli (separated by a distance of 3 cm) were grown simultaneously for 8 weeks wherein one set was Tat-bombarded and the second set was not, the transiently expressed Tat protein was extracellularly secreted (detected by a disc-blot immunoassay) by the Tat-bombarded calli (Fig. 2a). During week 1, the excreted Tat protein was found on one side of the culture dish, where the Tat-bombarded 14-day-old calli were located; gradually but steadily it migrated toward the other side until, by week 8, it completely filled up the entire 3-cm length between the two sets of calli (Fig. 2a). Control samples were also found to contain Tat from week 6 onwards, with a steady accumulation of Tat up to week 8 (Fig. 2b). These observations clearly demonstrate that extracellularly secreted Tat migrated towards the control calli and gained entry into the cells after 6–8 weeks. We therefore speculate that the presence of the RGD motif in Tat allowed Tat proteins to be secreted and to be absorbed by neighboring tomato tissues. It is worth mentioning that the steady accumulation of Tat in the control system that started from week 6 was enough to impair growth and development and culminate in plant death.

Another motif in Tat is the Arg-rich motif, which is known to be involved in binding to its RNA target (Meinhardt et al. 2002). In plants, however, there is a twin-Arg translocation (TAT) pathway which operates in the thylakoid membrane of chloroplasts. The main function of this twin-Arg translocation pathway is to transport fully folded proteins across the thylakoid membrane when a twin-Arg (RR) motif is found within the protein (Di Cola and Robinson 2005). The TAT pathway operates in the thylakoid membrane of chloroplasts, and the Arg-rich motif found in Tat contains an RR motif that may allow it to enter the chloroplast. Figure 2c shows the presence of Tat in the chloroplast, confirming Tat entry through the thylakoid membrane. Higher levels of CKO35 were also detected in the chloroplasts of Tat-bombarded samples than in those of the controls (Fig. 2d). Polanska et al. (2007) found that CK and CKO can be detected in both the cytoplasm and chloroplast. Our results suggest that CKO35 activity is increased in both the cytosol (Fig. 1d) and chloroplast (Fig. 2d), consequently reducing CK levels. This would also imply that the Arg-rich motif in Tat is responsible for both the entry of Tat into the thylakoid and the aberrant production of CKO35.

Fig. 2 Secretion, absorption and chloroplast entry of Tat and transduction of the Tat-associated phenotypes into adjacent healthy plants. **a** Tat secretion. Two sets of 14-day-old tomato calli were bombarded with pBI121 vector (*Control*) and pBI121-Tat (*Tat*), respectively. Both bombarded sets were concurrently grown together 3 cm apart for 8 weeks. Presence of chlorosis was indicated (*blue chevron*). Tat secretion was detected by disc-blot immunoassay using Tat-specific antibody. A Tat protein standard was made to estimate the amounts of Tat secreted. **b** Western blot assays demonstrating Tat expression in Tat-bombarded samples and the gradual absorption of secreted Tat in control samples. **c** Presence of Tat protein in tomato chloroplasts of Tat-bombarded samples. Week-1 and -8 tomato samples were analyzed by western blot assays. **d** Higher amounts of CKO35 were found in the chloroplasts of Tat-bombarded samples than in those of the controls ▶

Arg-rich motif of Tat produced the differentially expressed CKO35

We subjected both the Arg-rich and RGD motifs of Tat to in vitro mutagenesis, and the mutant clones were subsequently bombarded into 14-day-old calli concurrently with our original Tat construct and an empty vector. As shown in Fig. 3a, mArg (the mutated Arg-rich motif)-bombarded samples exhibited extracellular secretion but no chlorosis, while mRGD (the mutated RGD motif)-bombarded samples exhibited no extracellular secretion but showed initial signs of chlorosis. Figure 3b shows that the CK levels of the mArg samples are similar to those of the control, whereas mRGD samples have reduced CK levels that are comparable to those of the Tat samples. Furthermore, the CKO43 isoform was predominant in both the control and mArg-bombarded samples, while CKO35 was the only isoform detected in both the Tat- and mRGD-bombarded samples (Fig. 3c). Consistent with our previous observation (Fig. 1d), CKO37 was not detected since 14-day-old calli were used, and the shoot apical meristem, presumably where CKO37 is found, is not present at this stage. These results indicate that the Arg-rich motif is responsible for the differential expression of the CKO35 isoform, which affects CK levels, and that the RGD motif is responsible for extracellular secretion.


a
Week 1
Week 3
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Tat
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Tat standard
ng/μL
200
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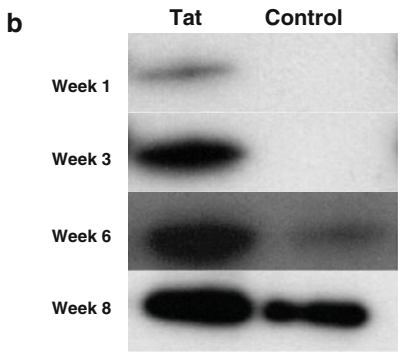
50
25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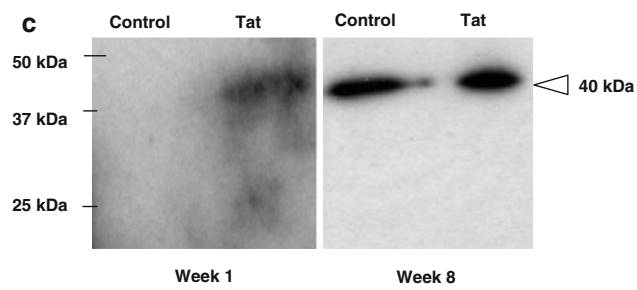
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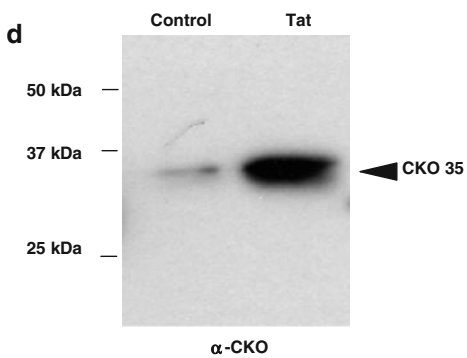
d
Control
Tat
50 kDa
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CKO 35
α-CKO

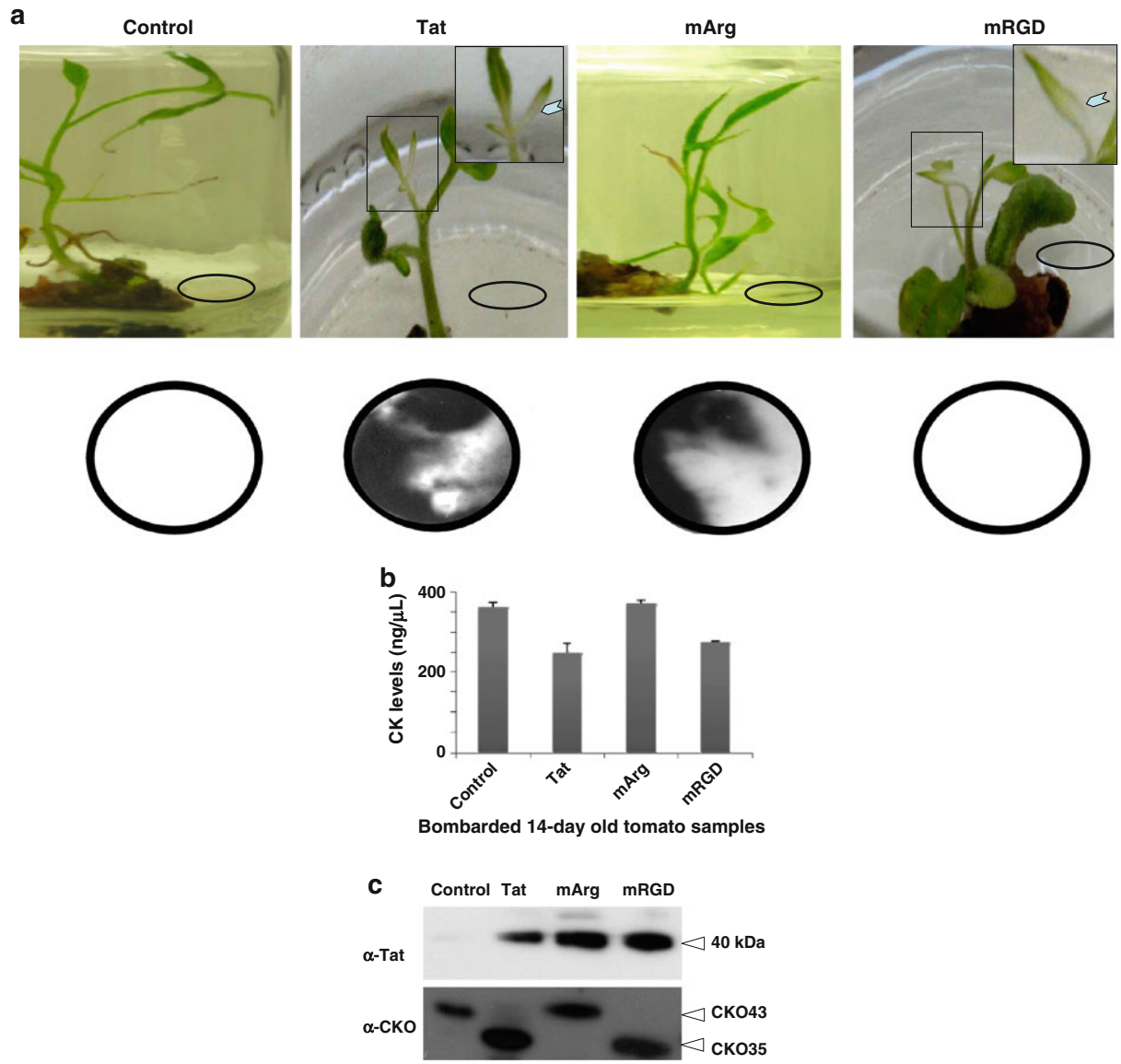

Fig. 3 Involvement of Arg-rich and RGD motifs in the effects of HIV-1 Tat on plant development. **a** Four sets of 14-day-old tomato calli were bombarded with an empty pBI121 vector (*Control*), pBI121-Tat (*Tat*), and the pBI121 vector expressing Tat mutated at the Arg-rich motif (*mArg*) or the RGD motif (*mRGD*). The presence of chlorosis is indicated (*blue chevron*), and Tat excretion was also detected through a disc-blot immunoassay using Tat-specific antibody (*right panels*). **b** CK levels were measured to confirm CKO activity. **c** Western blot assays verifying Tat and CKO expressions. Tat-specific and CKO-specific antibodies were used. CKO43 and CKO35 are indicated

An interesting observation was that mutation of the Arg-rich motif led to the development of normal plants with no chlorosis, suggesting that mutation of the Arg-rich motif may lead to the blocking of Tat protein transport across the thylakoid membrane and, thereby, disrupting Tat protein accumulation in the chloroplast. Mutation of the RGD motif prevented extracellular secretion but did not affect the transport of the Tat protein into the chloroplast; hence, there was chlorosis in the 14-day-old CDI tomato plantlets.

In conclusion, the results of this study provide answers the fundamental question on how HIV-1 Tat

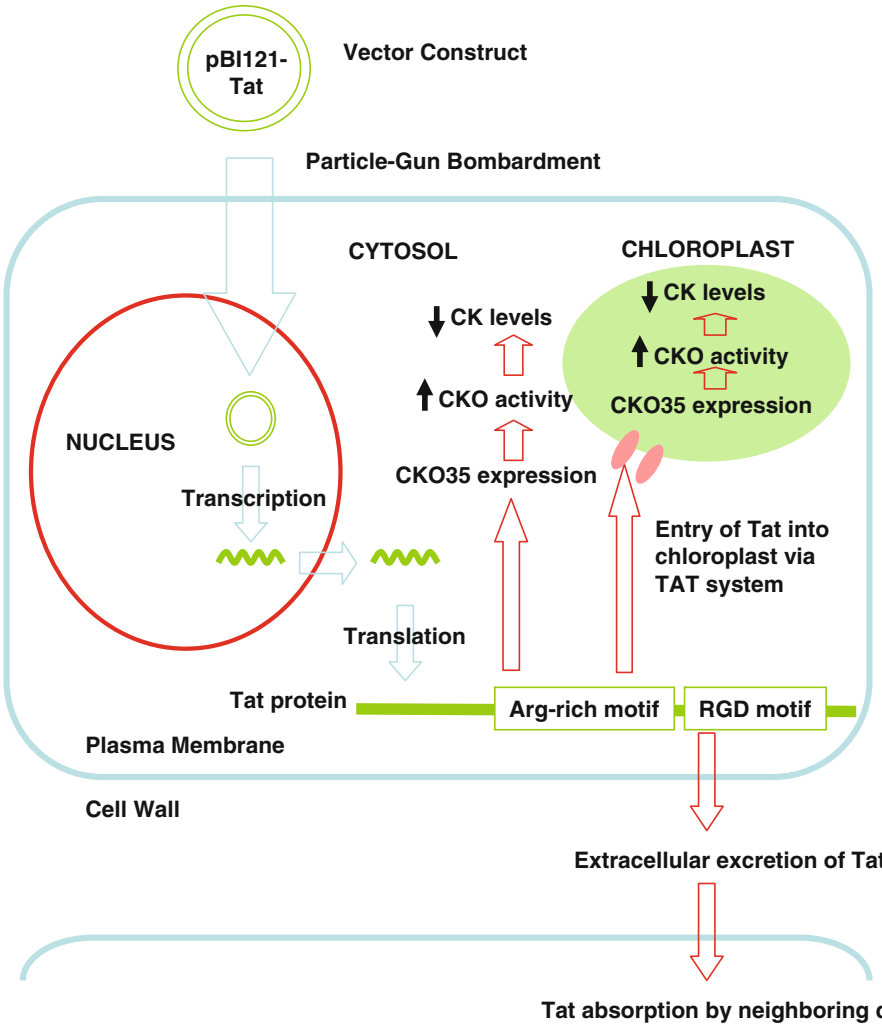

Fig. 4 Schematic diagram of the effects of HIV-1 Tat in a general plant system. Tat induces expression of a novel CKO species, CKO35, which reduces CK levels, thus causing the phenotypic abnormalities. The Arg-rich motif of Tat appears to be responsible for these abnormalities

protein expressed in a plant system affects the plant host (Fig. 4) and, concurrently, provides a new perspective in our understanding of Tat activity. At the enzymatic level, Tat appears to induce aberrant expression of the CKO35 isoform—in effect reducing CK levels and causing phenotypic abnormalities, such as stunted growth, absence of root formation, and abnormal chloroplast development leading to chlorosis. At the molecular level, the presence of the Arg-rich motif and RGD motif in Tat are both shown to allow Tat to enter the chloroplast via the chloroplast TAT system and to be extracellularly secreted, respectively. Likewise, mutation of the two functional motifs in Tat resulted in two distinct types of tomato plants, with mArg-bombarded tomato plantlets showing no change in CKO size and no apparent chlorosis but exhibiting extracellular secretion, and mRGD-bombarded tomato plantlets showing a change in CKO size and early signs of chlorosis but no extracellular excretion. Our results also demonstrate the potential for utilizing Tat or its two motifs in the plant-based protein production of pharmaceutical relevance.

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