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RESEARCH PAPER

Heterologous expression analyses of rice *OsCAS* in *Arabidopsis* and in yeast provide evidence for its roles in cyanide detoxification rather than in cysteine synthesis *in vivo*

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Abstract

While most dicot plants produce little ethylene in their vegetative stage, many monocots such as rice liberate a relatively large amount of ethylene with cyanide as a co-product in their seedling stage when etiolated. One of the known functions of β-cyanoalanine synthase (CAS) is to detoxify the co-product cyanide during ethylene biosynthesis in higher plants. Based on a tryptic peptide sequence obtained from a partially purified CAS activity protein preparation in etiolated rice seedlings, the full-length putative rice CAS-encoding cDNA sequence (OsCAS), which is homologous to those *O*-acetylserine sulphydrylase (*OASS*) genes, was cloned. Unlike most of the *CAS* genes reported from dicots, the transcription of *OsCAS* is promoted by auxins but suppressed by ethylene. To address the function and the subcellular localization of this gene product *in planta*, a binary vector construct consisting of this gene appended with a yellow fluorescent protein-encoding sequence was employed to transform *Arabidopsis*. Specific activities on CAS and OASS of the purified recombinant protein from transgenic *Arabidopsis* were 181.04 μmol H_2S mg^{-1} protein min^{-1} and 0.92 μmol Cys mg^{-1} protein min^{-1}, respectively, indicating that OsCAS favours CAS activity. The subcellular localization of OsCAS was found mostly in the mitochondria by immunogold electron-microscopy. Chemical cross-linking and in-gel assay on a heterodimer composed of functional and non-functional mutants in a yeast expression system on OsCAS suggested that OsCAS functions as a homodimer, similar to that of OASS. Despite the structural similarity of OsCAS with OASS, it has also been confirmed that OsCAS could not interact with serine-acetyltransferase, indicating that OsCAS mainly functions in cyanide detoxification.

Key words: Cyanide, β-cyanoalanine synthase, ethylene, rice, serine acetyltransferase.

Introduction

Cyanide is a toxic molecule generated in plants either by hydrolysis of cyanogenic glycosides in 'cyanogenic' species or from oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) during phyto-hormone ethylene biosynthesis. Most of the plants do not contain cyanogenic glycosides, but most of the higher plant species generate cyanide along with ethylene from ACC by the action of ACC oxidase (ACO) (Yip and Yang, 1988). Cyanide exerts its toxicity by inhibiting vital functions in cells including respiration (Grossmann, 1996; Solomonson, 1982).

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylate oxidase; At, *Arabidopsis thaliana*; BS^3, bis(sulphosuccinimidyl) suberate; CAS, β-cyanoalanine synthase; GFP, green fluorescent protein; HIS, consecutive histidine residues; OASS, *O*-acetylserine sulphydrylase; Os, *Oryza sativa*; PLP, pyridoxal-5′-phosphate; SAT, serine acetyltransferase; Sp, spinach; Wt, watermelon; YFP, yellow fluorescent protein.

Cyanide detoxification in plants can be handled by two enzymes including β-cyanoalanine synthase (CAS, EC 4.4.1.9) and rhodanese (EC 2.8.1.1). CAS has been demonstrated widely in higher plants (Blumenthal *et al.*, 1968; Miller and Conn, 1980), insects (Ogunlabi and Agboola, 2007), and bacteria (Dunnill and Fowden, 1965; Castric and Strobel, 1969); while rhodanese has been found from animals sources (Jones, 1998), bacteria (Westley, 1973), and also in a few higher plant species (Tomati *et al.*, 1972; Chew, 1973). When feeding higher plants with either ^{14}C-labelled cyanide or ^{14}C-labelled ACC at C-1, radioactive carbon would first be incorporated into β-cyanoalanine and then converted to asparagine by a hydratase (Peiser *et al.*, 1984). Later, Yip and Yang (1988) demonstrated the importance of CAS in cyanide metabolism during active ethylene biosynthetic conditions such as in fruit ripening, organ senescence, auxin-induction, and in various stress conditions, etc. Thus, it is widely accepted that cyanide detoxification in higher plants is mainly by CAS activity.

Purification and kinetic studies on CAS from plants were carried out by Hendrickson and Conn (1969). They demonstrated that CAS is a pyridoxal-5′-phosphate (PLP)-dependent enzyme which also possesses cysteine synthase activity but favours CAS activity. By contrast, a closely related group of PLP-dependent *O*-acetylserine sulphydrylases (OASS: EC 4.2.99.8) are homologous with CAS (Hatzfeld *et al.*, 2000; Maruyama *et al.*, 2001), but favours cysteine synthase activity (equivalent to OASS activity) and also CAS activity. That a specific lysine residue facilitates PLP binding on spinach OASS was determined by complementation in cysteine-deficient bacteria with SpOASS mutants on the lysine residue (Saito *et al.*, 1993). Hence, it is highly possible that the same lysine residue on CAS protein may form a Schiff base, facilitating the β-replacement in the CAS reaction.

Subcellular localization of various OASS has been found in organelles including cytosol, chloroplast, and mitochondrion in plants by immuno-detection on protein isolated from subcellular fractionation in spinach (Takahashi and Saito, 1996) and *Arabidopsis* (Hesse *et al.*, 2004), *in vitro* import assay to mitochondria in *Arabidopsis* (Hesse *et al.*, 2004), or OASS activity assay on extracts from different subcellular fractionations in *Datura innoxia* Mill. (Kuske *et al.*, 1996). CAS has been determined in mitochondria exclusively by mitochondrial fractionation in barley leaves (Wurtele *et al.*, 1985) and blue lupin seedlings (Akopyan *et al.*, 1975). Due to the main difference in subcellular localizations among these two proteins, it is hypothesized that CAS should localize better to mitochondria for effective removal of cyanide so as to protect the oxidative phosphorylation process; and that the optimal pH for CAS activity is around 8.5 which is also the pH in the matrix of mitochondria. Some authors have suggested that CAS could be regarded as OASS-like protein located in mitochondria, but in *Arabidopsis* two different genes coding for CAS and OASS co-exist in the mitochondria (Jost *et al.*, 2000). Therefore, it is inappropriate to assign CAS and OASS to a subcellular location based on activity assays and immunoassays on the subcelluar fractions, as both types of proteins possess overlapping structural and functional properties. A better approach to determine their subcellular location in plant cells would be to visualize OASS/CAS fused with a fluorescent protein.

Beside the overlapping structural and functional properties of CAS and OASS, both proteins are supposed to work in sequence *in vivo.* CAS removes cyanide by combining it with cysteine to form β-cyanoalanine, the displaced sulphide being recycled back to cysteine by the action of OASS. It is widely accepted that cysteine synthesis is a highly regulated process that is catalysed by a cysteine synthase complex comprising of serine acetyltransferase (SAT) and OASS in bacteria and plants (Kredich, 1971; Nakamura *et al.*, 1987; Droux *et al.*, 1992, 1998; Liszewska *et al.*, 2005). It is not known whether CAS could also interact with SAT, and whether certain OASS/CAS gene family member(s) could possess a dual function *in vivo.* Based on the OASS/CAS mutant study in *Arabidopsis*, K Saito's group speculated that some OASS proteins in cytoplasm can function in cyanide detoxification (Watanabe *et al.*, 2008).

Although the importance of CAS in cyanide detoxification during ethylene biosynthesis has been postulated for almost two decades (Yip and Yang, 1988), there have been very few studies on CAS in plant species with high ethylene biosynthetic rate except one recently on apple fruit (Han *et al.*, 2007). A high rate of ethylene biosynthesis (2 nmol g^{-1} h^{-1}) is found in rice seedlings under etiolated growth; this is comparable to ripening apple (2–5 nmol g^{-1} h^{-1}). In these tissues, ample capacity of CAS activity was also found. By following the CAS activity in the etiolated rice (*Oryza sativa*) seedlings with a chromatograph scheme and based on a tryptic sequence of the partially purified CAS preparation, it has been possible to isolate *OsCAS* used in this study. The aim is to establish the relevance of this gene-encoding protein in cyanide detoxification in rice. By visualizing the recombinant OsCAS protein expressed in *Arabidopsis*, the evidence that *OsCAS* encodes an authentic CAS is provided here by satisfying three consensus criteria: (i) a high CAS to OASS activity ratio with mM to sub-mM range K_m for cyanide (Hatzfeld *et al.*, 2000; Maruyama *et al.*, 2001; Han *et al.*, 2007); (ii) a severe inhibition on CAS activity at [HCN] above 10 mM (Jost *et al.*, 2000; Warrilow and Hawkesford, 2000); and (iii) CAS should localize to mitochondria (Wurtele *et al.*, 1985). To delineate CAS from OASS in the OASS/CAS gene families further, chemical cross-linking and mutagenesis experiments have been conducted on the products of these genes. The native assembly and amino acid residues important for OsCAS activity were examined.

Materials and methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*; Columbia ecotype 0) plants were allowed to germinate on MS (Murashige, 1962)

agar plates with an antibiotic selection. Resistant seedlings were transferred to soil and kept in a growth chamber at 22 °C under a 16/8 h photoperiod at 264 μmol m^{-2} s^{-1}. After 3 weeks, leaves were harvested at a stage before flowering and material was used for identification of *OsCAS* transgenic lines and confirmation of authentic OsCAS protein function in these transgenic lines.

RNA extraction and the northern blotting on transgenic lines

Total RNA was extracted using the hot phenol extraction method (Katharina, 1995). Twenty micrograms of total RNA was separated in a 1.2% formaldehyde gel in MOPS buffer pH 7 and 5% formaldehyde and then blotted onto a piece of Hybond-N^+ membrane (GE Healthcare). After cross-linking under an ultraviolet lamp, the blot was prehybridized with denatured salmon sperm DNA at 42 °C for 6 h with rotation. During prehybridization, radioactive probe was synthesized *in vitro* by using the Random Primed DNA Labelling Kit (Roche) with radioactive [^{32}P]dCTP following the manufacturers' instruction. The purified denatured probe was added on the blot with rotation overnight. After washing steps, the blot with an X-ray film (Fujifilm) was locked in a cassette and this cassette was kept in –80 °C for several days. Film was developed by an X-ray processor (Kodak X-OMAT).

Genomic DNA extraction from transgenic lines and Southern blot analysis

Genomic DNA was extracted from 3 g of leaves from transgenic lines with cetyltrimethylammonium bromide (Allen *et al.*, 2006) and then digested with the two restriction enzymes *Sac*I and *Eco*RV at 37 °C overnight. After agarose gel electrophoresis at a constant 40 V, digested genomic DNA in gel was denatured in alkali prior to blotting. Probe synthesis with radioactive labelling, hybridization, and imaging were as described in the preceding section.

Protein extraction from Arabidopsis *transgenic lines*

Leaves were ground in liquid nitrogen and resuspended in extraction buffer containing 50 mM TRIS-HCl (pH 8.5), 5 mM EDTA, 10 μM pyridoxal-5-phosphate, and 30% glycerol, the homogenate was kept on ice for 2 min with vortex and centrifuged at 17 900 *g* for 10 min sequentially. The supernatant was filtered through two layers of nylon net filters NY80 (Millipore) and the filtrate was saved for enzyme activity determination and OsCAS–yellow fluorescent protein (YFP) purification. All steps were performed at 4 °C unless otherwise specified.

Measurement of ethylene production from rice seedlings by gas chromatography

Ten rice seedlings (~3 cm from apex of seedling) were harvested and collected in a sealed 6 ml glass tube filled with 200 μl water. The tube was incubated either in light or dark for 1 h and then 1 ml of gas from the tube was injected into the gas chromatography (Hewlett-Packard 5890 Series II; Hewlett-Packard, Wilmington, DE, USA) according to Yip and Yang (1988). The concentration of ethylene was presented in parts per million (ppm) and the rate of ethylene production from seedlings was determined as a unit of nmol g^{-1} h^{-1}.

N-terminal protein sequencing and protein identification by tryptic mass fingerprinting

N-terminal sequencing of mature OsCAS–YFP protein from transgenic lines was conducted on a protein sequencer (Hewlett-Packard G1000A). Purified OsCAS–YFP protein separated in 10% SDS–PAGE was blotted on Immobilon™-P (Millipore) according to the user guide from Millipore and then the membrane was stained in Coomassie blue. The protein band of OsCAS–YFP was excised and saved for N-terminal protein sequencing.

Immuno-precipitated protein bands were identified by tryptic mass fingerprinting according to Luk *et al.* (2005). Peptide masses were determined by a MALDI–TOF MS (Voyager-DE™ STR Biospectrometry™ Workstation; Applied Biosystems, Foster City, CA, USA). Peptide mass lists were searched against the online database of the National Center for Biotechnology with limitation on species by program MS–FIT (http://prospector.ucsf.edu). Program parameters were selected as follows: monoistopic peptide masses adopted, mass tolerance set as 100 ppm, and allowance of one missed cleavage.

In-gel CAS activity assay, supershift in gel CAS activity assay, CAS activity assay, OASS activity assay, and SAT activity assay

Twenty micrograms of protein extract from *Arabidopsis* transgenic lines were incubated with a series dilution of polyclonal anti-green fluorescent protein (GFP) antibodies (Invitrogen) on ice for 30 min and then centrifuged at 17 900 *g* for 1 min for supershift in-gel CAS activity assay or no antibody was mixed with protein extract from these lines for original in-gel CAS activity assay. Supernatants were separated in a 10% native PAGE. The in-gel CAS activity assay in slab gel was conducted, based on the methods described by Akopyan *et al.* (1975), after electrophoresis, except CAS activity staining was done at 35 °C for 30 min. CAS activity and OASS activity assays were performed as described by Yip and Yang (1988) and Lunn *et al.* (1990), while the SAT activity assay was conducted following a spectrophotometric assay of Bonner *et al.* (2005). Determination of enzymatic activities was repeated at least in triplicate.

Protein purification and quantification of OsCAS–YFP from Arabidopsis *transgenic lines*

Native purification of OsCAS–YFP was conducted using uMAC™ epitope tag protein isolation kit with polyclonal anti-GFP microbeads (Miltenyl Biotec) following the

manufacturer's instructions. To perform each immunopurification, protein extract from 1 g of leaves was required. Protein extract was incubated with anti-GFP microbeads in ice for 30 min and the protein–antibody bead complex was loaded in a column under a strong magnetic force. After column washing steps, purified OS CAS–YFP was eluted in native elution buffer containing 50 mM TRIS-HCl (pH 8.5), 5 mM EDTA, 10 μM PLP, and 30% glycerol without magnetic interaction. The brown eluate was collected for CAS activity assay or denative electrophoresis.

Protein quantification of pure OsCAS–YFP in eluate was estimated by a PharoxFX™ molecular imager system (BIO-RAD), based on Coomassie blue staining on SDS–PAGE with bovine serum albumin as a standard.

Immunoblotting

Protein samples from either total protein from *Arabidopsis* or purified OsCAS–YFP eluate were separated in a 10% (w/v) acrylamide gel by SDS–PAGE (Laemmli, 1970). Proteins were subsequently transferred to Hybond ECL nitrocelluolose membrane (Amersham Biosciences) using Hoefer Semiphor (Pharmacia Biotech) at a maximum current of 100 mA for 1 h. Rabbit polyclonal anti-GFP antibodies (Invitrogen) and donkey ECL horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Amersham Biosciences) were both applied at 1:5000 dilution. Signals were captured on a sheet of autoradiography film after incubation with detection reagents (Amersham Biosciences).

Generation of OsCAS–YFP overexpression Arabidopsis *transgenic lines*

A full-length CAS encoding sequence was amplified with primers 5′-GGATCCATGGAGAGGCTCTGATGAGCC-TCATGA-3′ and 5′-CTCGAGTTAGTCCACTGGCACT-GGCTGATGGCCT-3′ from pCR OsCAS and then cloned into pCR-Blunt II-TOPO vector. This full-length sequence was recovered by restriction enzyme digestion with *Sal*I and *Kpn*I and this fragment was subcloned into *Xho*I- and *Kpn*I-digested pBA002-EYFP vector. Expression of OsCAS tagged with EYFP at the C-terminal was driven by the CaMV 35S promoter in vector pBA002-EYFP. The construct was mobilized into *Agrobacterium tumefaciens* ABI strain by freeze–thaw transformation (Chen *et al.*, 1994) and introduced to *Arabidopsis* ecotype (Col-0) *in planta* by a floral dip (Clough and Bent, 1998).

Chemical cross-linking and immunoblotting of Os CAS–YFP complex in crude leaf extract

Various concentrations of bis(sulphosuccinimidyl) suberate (BS^3) (Pierce) were added to 2 mg ml^{-1} of *Arabidopsis* crude leaf protein extracted in conjugated buffer containing 100 mM sodium carbonate (pH 8.5), 5 mM EDTA, 10 μM PLP, and 30% glycerol. The cross-linking reaction was carried out at 0 °C for 2 h and was quenched subsequently by adding 50 mM TRIS (pH 8.0) for 30 min at room temperature. Cross-linked protein was precipitated from the reaction mixture by adding 1 volume of 20% of trichloroacetic acid for 45 min at –20 °C. After high-speed centrifugation at 35000 *g* for 15 min, protein precipitate was washed by 1 volume of acetone containing 1 mM phenylmethylsulphonyl fluoride and 2 mM EDTA twice and lyophilized for 1 h. Protein precipitate was dissolved in 1× electrophoresis sample buffer at room temperature overnight. A protein sample (40 μg $lane^{-1}$) was analysed in a 10% (w/v) acrylamide gel by SDS–PAGE (Laemmli, 1970). Proteins in PAGE were transferred to Hybond ECL nitrocelluolose membrane (Amersham Biosciences) and detected by a rabbit polyclonal anti-GFP antibody.

Cryo-EM immunolocalization

Immunogold labelling of *Arabidopsis* cells was carried out as described previously (Tse *et al.*, 2004). Briefly, transgenic *Arabidopsis* seeds (OsCAS–YFP line 4 and YFP line 1) were subjected to germination on an MS agar plate with BASTA for 3–4 d before root tips (about 0.2 cm in length) were collected and fixed in 2 ml of fixative solution containing 0.25% (v/v) glutaraldehyde and 1.5% (v/w) paraformaldehyde in 50 mM phosphate buffer, pH 7.4, for 15 min at room temperature, and then transferred to 4 °C for an additional 16 h. After washing with phosphate buffer at room temperature, cells were dehydrated in an ethanol series and then embedded in Lowicryl HM20 resin. Ultrathin sections were then prepared from the Lowicryl HM20 blocks, followed by blocked in blocking buffer PBS (80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride) with 3% BSA. Treated samples were then incubated with GFP antibodies (at 10 μg ml^{-1} diluted with PBS containing 1% BSA) for 1 h, followed by washing with buffer (PBS containing 1% BSA) before incubation with gold-conjugated secondary antibodies (at 1:50 diluted with PBS containing 1% BSA). Labelled samples were post-stained with uranylacetate before the stained specimens were examined in a JOEL JEM-1200EX II transmission electron microscope (JOEL, Tokyo, Japan) operating at 80 kV as described (Tse *et al.*, 2004). The GFP antibodies used in this study were prepared by injecting recombinant GFP into rabbits at the animal house of The Chinese University of Hong Kong and affinity-purified.

Protein expression in budding yeast

Recombinant protein was overexpressed in yeast transformant (strain INVSc1) with a pYES2 vector (Invitrogen) inserting a coding DNA sequence (for OsCAS expression, the N-terminal 35 amino acid residues encoding sequence was deleted). Protein expression was induced in minimal medium with galactose at 30 °C overnight with continuous shaking. Yeast cells were disrupted in breaking buffer containing 50 mM TRIS-HCl (pH 8.5), 5 mM EDTA, 10 μM PLP, and 30% glycerol by vortexing with glassbeads at 4 °C. These steps followed the procedures given in the manufacturer's manual (Invitrogen).

Miscellaneous molecular techniques

A list of miscellaneous molecular techniques including polymerase chain reaction, restriction enzyme digestion, ligation, and plasmid transformation into *Escherichia coli*, etc. were conducted according to Sambrook and Russell (2001). Silver staining on SDS–PAGE was carried out following the Vorum silver-staining method (Mortz *et al.*, 2001), while recombinant His-tagged protein in yeast was purified by a nickel column according to the instructions in the manufacturer's manual (Invitogen).

Results

Alignment of the amino acid sequence of OsCAS with those of CAS in other plant species

By following the increase in CAS activity through chromatography (starting from 31.02 μmol min^{-1} to 4.56 μmol min^{-1} after Mono Q column with a purification fold of 98%), CAS proteins were successfully purified in near homogeneity from etiolated rice seedlings. A tryptic peptide (LIVTVLPSLGER), a digestion product from this protein on a denative 2-D gel, could be resolved by MS/MS equipped with a nanospray source. Searching the database from the translated rice genome, this dodecapeptide was found to be a complete match with a protein encoded by a putative rice CAS gene (*OsCAS*) but not with other members in the rice OASS/CAS gene family. This putative OsCAS also contained a highly conserved PLP-binding domain (PXXSV/IKDR) as do other members in the rice OASS/CAS gene family in which the lysine residue may form a Schiff base with the PLP co-factor. By using primers according to the nucleotide sequence in the database, a full-length 1134 bp cDNA encoding OsCAS was amplified by PCR. The gene-encoding sequence and protein sequence of OsCAS have been deposited on Genbank® with accession numbers AY720933 and AAV48542, respectively. This translated protein consists of 378 amino acids and its sequence shows 69.3–71.2% homology with those CAS sequences from other plant species in the polypeptide alignment (Fig. 1). The N-terminal amino acid sequence of the mature OsCAS protein harvested from those OsCAS heterologous expression *Arabidopsis* lines has been sequenced by Edman degradation. The result revealed that the first 35 amino acid residues from the N-terminal of OsCAS were cleaved (data not shown), hence the molecular mass of the mature OsCAS monomer is 36.5 kDa. This cleaved peptide possibly serves as the mitochondrial targeting peptide based on the result of EM immunolocalization described in the following results.

Expression of OsCAS *transcript in etiolated seedlings under different treatments*

Yip and Yang (1988) demonstrated the importance of CAS in cyanide metabolism during active ethylene biosynthesis based on elevation of the cyanide level in plant tissues incubated with both the ethylene precursor (ACC) and CAS inhibitor (aminooxyacetic acid). CAS acts downstream of ACC synthase and ACO to remove the co-product cyanide. Auxins are known to induce ethylene biosynthesis by promoting ACC synthase (Kim *et al.*, 1992) and ACO gene transcription (Petruzzelli *et al.*, 2000); while ethylene has been shown to have both promotion and inhibition effects on gene transcription in some members of these two gene families. Hence, auxins and ethylene effects on *OsCAS* transcription were examined. It was found that ethylene biosynthesis in etiolated rice seedlings could be further induced by infiltration with 10 mM 2,4-D (a synthetic auxin). The ethylene biosynthetic rate was induced to about 7-fold over the control in seedlings treated with 2,4-D while the rate was suppressed to a non-detectable level in seedlings treated with cobalt chloride (an inhibitor of ACO), and with both 2,4-D and cobalt chloride (Fig. 2A, upper panel). The *OsCAS* transcripts from seedlings with different treatments were analysed by northern blotting with ^{32}P radioactive *Xho*I–*OsCAS*–*Xho*I probe. The *OsCAS* transcript from untreated seedlings was low but detectable, it was greatly induced in seedlings treated with 2,4-D and 2,4-D plus cobalt chloride, although the ethylene biosynthetic rates of the seedlings in these treatments were completely different (Fig. 2A, lower panel). The *OsCAS* transcripts from seedlings treated with cobalt chloride were induced slightly despite a drop of ethylene biosynthetic rate to a non-detectable level.

The increase in *OsCAS* expressions in rice seedlings could be related to a decline in endogenous ethylene biosynthesis. Rice seedlings, etiolated for 7 d, were transferred from dark to light in a constant temperature (25 °C). The seedlings were harvested to determine the ethylene biosynthetic rates and analyse *OsCAS* transcripts at 2 h intervals. The ethylene biosynthetic rates of these seedlings declined from about 2 nmol g^{-1} h^{-1} at 0 h to a non-detectable level at 4 h and the rate stayed at this level until 8 h (Fig. 2B, upper panel). The accumulation of *OsCAS* transcripts in rice seedlings was elevated accompanying the decline of ethylene biosynthetic rates. The transcripts began to accumulate from 4 h when the ethylene biosynthetic rate in the seedlings reached a non-detectable level (Fig. 2B, lower panel).

Generation of OsCAS–YFP overexpression in Arabidopsis *and studies of CAS activity in transgenic lines*

To facilitate studies on enzyme kinetics, structural properties, and subcellular localization of *OsCAS* encoding protein, *Arabidopsis OsCAS–YFP* overexpression lines (OsCAS–YFP lines) were generated. Expression of a 1.1 kb full coding sequence of *OsCAS* without a stop codon appended with a 0.8 kb YFP coding sequence was driven by a CaMV promoter. For the control transgenic line (YFP line), a 0.8 kb YFP coding sequence was driven by the CaMV promoter (Fig. 3A). Seeds from these lines were allowed to germinate on MS basal agar plates (Murashige and Skoog, 1962) under BLASTA selection and then

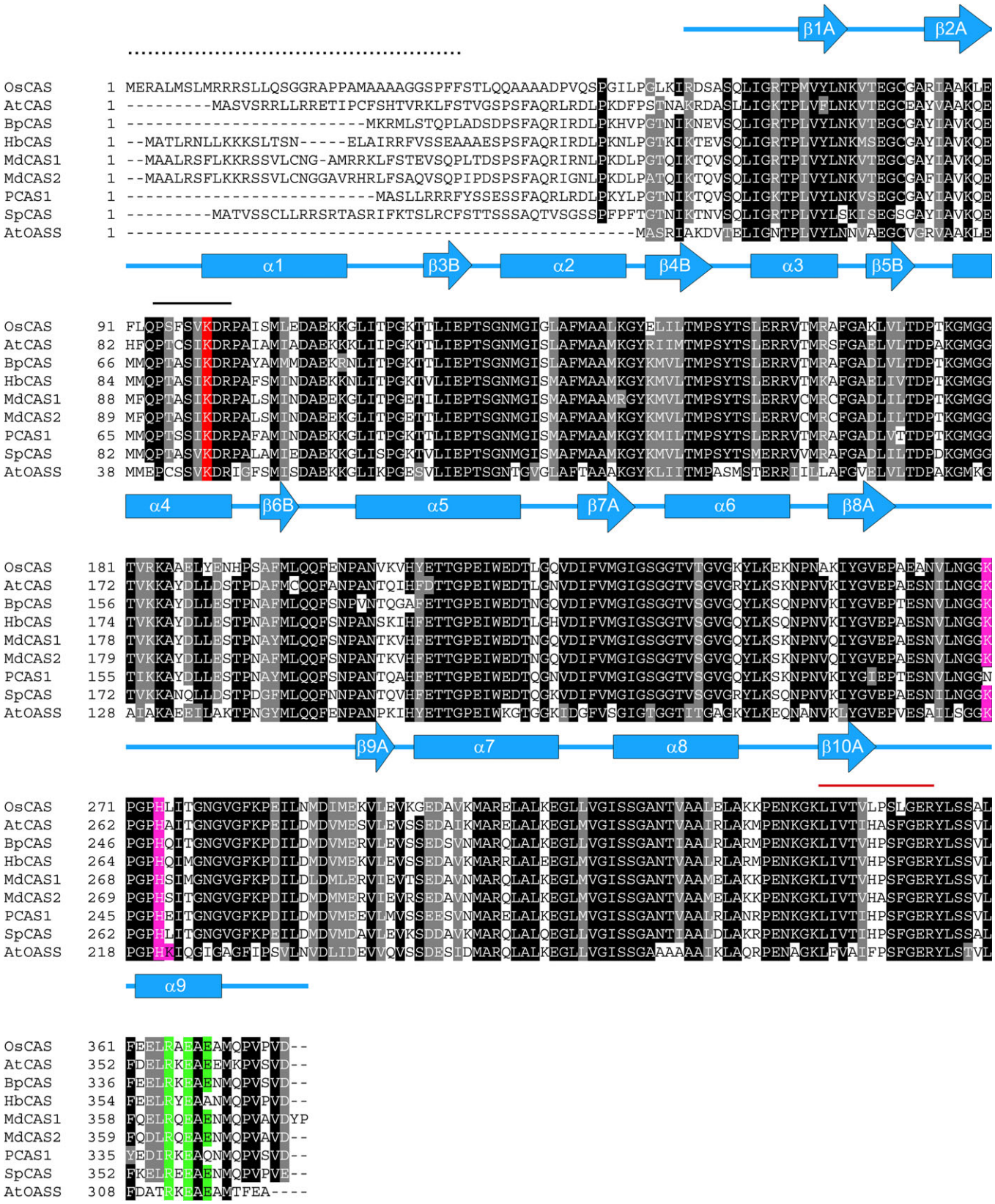

Fig. 1. Alignment of sequences of OsCAS and other CAS from plant species with AtOASS from *Arabidopsis*. Comparison of the derived amino acid sequence of OsCAS was made with those of the *Arabidopsis* AtCAS (Hatzfeld *et al.*, 2000), spinach SpCAS (Hatzfeld *et al.*, 2000), potato PCAS1 (Maruyama *et al.*, 2001), birch BpCAS (GeneBank accession number AY154650), latex (*Hevea brasilienis*) HbCAS (GeneBank accession number AAP41852), Fuji apple MdCAS1, MdCAS2 (Han *et al.*, 2007), and *Arabidopsis* AtOASS (EMBL number CAA56593). Amino acid residues that are conserved in at least eight of the nine sequences are shown in black. Secondary structures of AtOASS are represented in α-helices (blue rectangles) and β-stands (blue arrows). The solid line in black shows the conserved pyridoxal

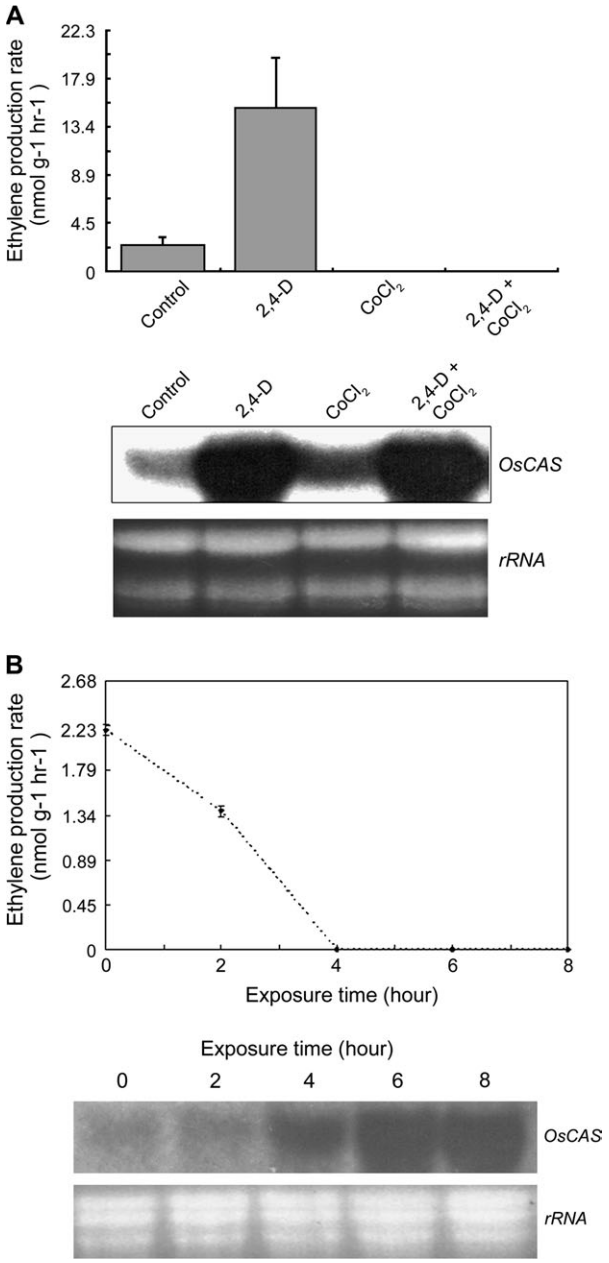

Fig. 2. Ethylene production rates and expressions of *OsCAS* transcripts in etiolated rice seedlings with 2,4-D, cobalt chloride, or both treatments. Ethylene biosynthetic rates on rice seedlings treated with different chemicals at 6 h after treatments (A), or transferred from dark to light (B) (upper panel). Expressions of OsCAS transcripts from seedlings were analysed by northern blotting with the ^{32}P radioactive-labelled OsCAS probe (lower panel). Twenty micrograms of the total RNAs from seedlings were loaded per lane to show the equal loading. Measurement of ethylene production and the northern blotting of the hormone-treated sample were conducted in triplicate.

resistant seedlings were transplanted into soil. Leaves from different lines were harvested 1 week after transplantation. Successful transformation was confirmed by genomic Southern blotting with either ^{32}P-labelled *YFP* or the *Xho*I–*OsCAS*–*Xho*I probe (Fig. S1 in Supplementary data available at *JXB* online).

The expression levels of *OsCAS*–*YFP* and *YFP* transcripts were analysed in these lines. Twenty micrograms of total RNA from *Arabidopsis* leaves were separated in a 1.5% formaldehyde gel. Two different regions from constructs including 0.7 kb *Xho*I–*OsCAS*–*Xho*I from pBA OsCAS–YFP and 0.8 kb *Xho*I–*YFP*–*Spe*I from pBA YFP were selected as probes for quantification of the expression levels of these transcripts. Northern blotting on these OsCAS lines (Fig. 3B, lanes 1–5) showed that a 1.76 kb transcript of *OsCAS*–*YFP* was detected with various expression levels by hybridization with the ^{32}P-labelled *Xho*I–*OsCAS*–*Xho*I probe, except the YFP line (Fig. 3B, lane 6). The northern blot analysis by hybridization to ^{32}P-labelled *Xho*I–*YFP*–*Spe*I probe showed that the presence of YFP transcripts, 0.72 kb in size, in the YFP line. The northern blot analysis confirms that the transcripts of *OsCAS*–*YFP* and *YFP* are expressed in OsCAS–YFP lines and YFP line, respectively.

To verify the CAS activity band due to heterologous expression of OsCAS in these transgenic lines, in-gel CAS activity assay was conducted. An extra CAS activity band was found in OsCAS–YFP lines compared with the YFP line (Fig. 3C). A strong enzyme-stained band in all lines denoted as endogenous *Arabidopsis* CAS activity while the uppermost CAS activity-stained band is possibly due to the protein encoded by OsCAS–YFP (Fig. 3C, lanes 1–5). This additional band was detected in all OsCAS–YFP lines, and OsCAS–YFP line 4 was selected for downstream experiment for its strong CAS activity.

To confirm that this new CAS activity band was due to OsCAS–YFP in these transgenic lines, 'super-shift in-gel CAS activity assay' was conducted on crude leaf extract from OsCAS–YFP line 4. This enzyme assay method is similar to the in-gel CAS activity assay except that incubation was with different dilutions of polyclonal anti-GFP antibody prior to sample separation in the non-denative polyacrylamide gel. In-gel CAS activity assay was analysed on crude leaf extract from OsCAS–YFP line 4 incubated with different concentrations of polyclonal anti-GFP antibody (Fig. 3D). Intensities of the free form of CAS (Fig. 3D; indicated across the top, labelled OsCAS–YFP) decreased gradually when incubated without antibody (Fig. 3D, lane 9) to with antibody in a dilution of 1/10240. This free form was completely shifted to an upper position at antibody dilutions of 1/5120 and 1/7680. The decrease in

5′ phosphate binding site while the red line covers a peptide from the partially purified rice CAS fraction sequenced by MS/MS. The mitochondrial target sequence from OsCAS which was determined by N-terminal Edman degradation on a mature OsCAS protein purified from a heterologous expression of *Arabidopsis* plant is indicated by dots. Amino acid residues which were chosen in this mutagenesis study are highlighted: Lysine in the PLP-binding domain is shaded in red while conserved amino acid residues for SAT interaction and dimerization are shaded in purple and green, respectively.

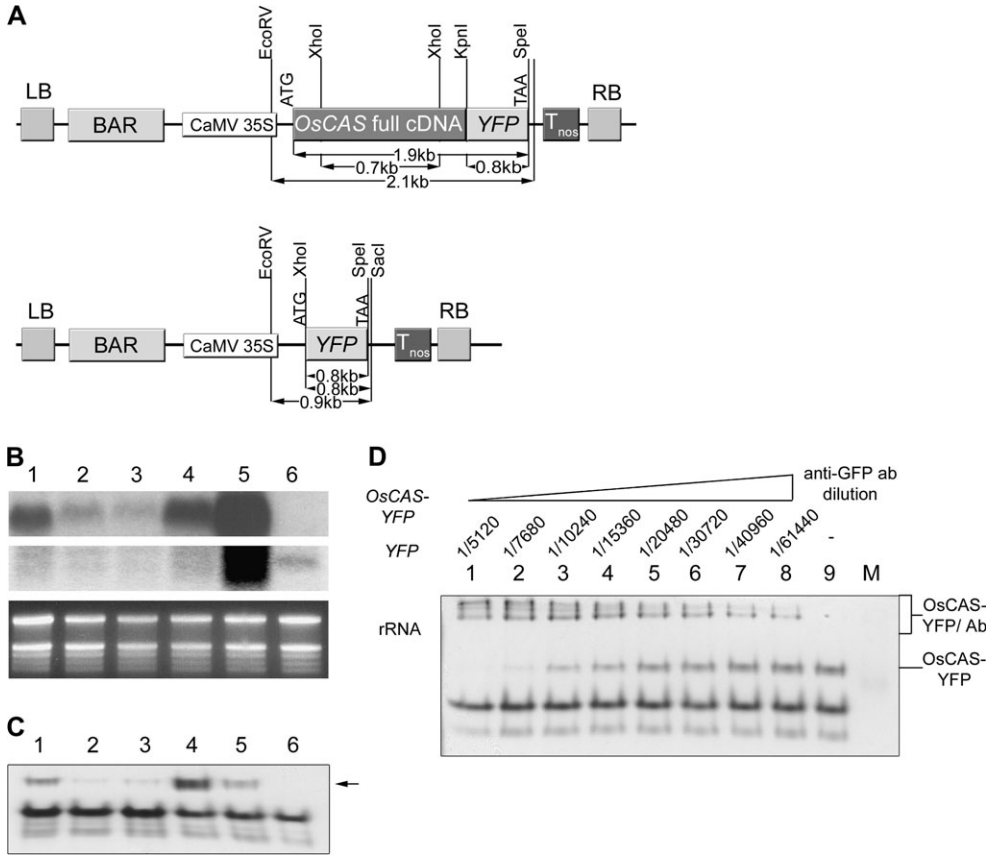

Fig. 3. Expression of OsCAS in its heterologous expression *Arabidopsis* lines. (A) Schematic diagram of the binary vectors pBA OsCAS–YFP and pBA YFP. LB and RB refer to the left and right borders of the T-DNA, respectively. CaMV35S, Cauliflower mosaic virus 35S promoter; T_{nos}, terminator of nopaline synthetase gene; BAR, gene conferring BLASTA resistance. (B) Transcripts of *OsCAS* and *YFP* were analysed from 20 μg total RNA from leaves of these transgenic lines (lanes 1–5) and YFP line (lane 6) with OsCAS (first panel) or YFP probe (second panel), revealed by northern blotting. (C) An in-gel CAS activity assay was performed on 20 μg of leaf extracts from OsCAS–YFP transgenic lines (lanes 1–5) and YFP line (lane 6). A strong additional CAS activity band (arrowed) was found in the OsCAS–YFP transgenic line 4. To elucidate this additional CAS band due to OsCAS–YFP further, 20 μg of leaf proteins from line 4 were applied in a supershift in-gel assay (D). Ab, Antibody.

intensity of free form CAS was accompanied by an increase in intensity of the complex form (Fig. 3D; indicated across the top, labelled OsCAS–YFP/anti-GFP ab dilution). Three complexes of different molecular mass were observed in crude extracts incubated with antibody in 1/5120 to 1/10240 dilutions. Hence, by selecting OsCAS–YFP line 4 as a representative, this assay confirmed that OsCAS protein from these lines possesses CAS activity *in vitro*.

Immuno-pull down of OsCAS–YFP from the transgenic line and kinetic studies on the immuno-purified protein

To characterize the enzyme activity of OsCAS–YFP protein *in vitro*, it was purified from a crude extract from leaves of transgenic line 4 by employing immuno-pull down with anti-GFP antibody immobilized on metal beads with washing by native extraction buffer. The pull-down proteins from the transgenic OsCAS–YFP line 4 and YFP line were separated in a 10% PAGE and visualized by silver staining. Two protein bands with molecular masses of 64 kDa and 28 kDa, respectively, were observed in the protein extract from OsCAS–YFP line 4 compared with the YFP line (Fig. 4A); these bands cross-reacted with anti-GFP antibody (Fig. 4B). An almost identical protein pattern was observed in pull-down protein from the transgenic OsCAS–YFP line 4 with strong stringency washing buffer. Four proteins with different molecular masses from this pull-down fraction were separated in a 10% PAGE (Fig. 4C) and three of these were identified by searching the molecular masses of tryptic-digested peptides from a protein against the database of translated rice genome encoding sequences. These three proteins with molecular masses about 64, 52, and 28 kDa were beta-cyanoalanine synthase (accession no. AY720933) with a molecular mass of 40 kDa, a large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (accession no. BAA84393) with a molecular mass of 53 kDa, and a green fluorescent protein RSGFP variant (accession no. AAA79304.1) with a molecular mass of 29 kDa, respectively (Table S1 in Supplementary data available at *JXB* online). The identity of the protein with a molecular mass 64 kDa should be OsCAS–YFP as the result of database searching and cross-reactivity with anti-GFP antibody, while the

protein with a molecular mass 25 kDa was possibly the light chain of anti-GFP antibody (Fig. 4D). In this pull-down experiment, it was revealed that among these proteins OsCAS–YFP was in the majority while the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and YFP were in the minority. By contrast, YFP is a unique band in an immuno-precipitated protein from the YFP transgenic line 1.

CAS/OASS activity and kinetic parameters with different substrates on immuno-purified OsCAS–YFP were determined on purified OsCAS–YFP protein from *OsCAS–YFP* overexpression transgenic line 4 according to the enzyme assay methods for CAS from Yip and Yang (1988) and for OASS from Lunn *et al.* (1990) (Table 1). The specific enzyme activity of CAS on OsCAS–YFP was calculated as 181 μmol H_2S min^{-1} mg^{-1} protein, while that of OASS on OsCAS–YFP was 0.92 μmol Cys $min^{-1}mg^{-1}$ protein. OsCAS–YFP showed much stronger activity of CAS than that of OASS with a ratio of 197. Regarding kinetic parameters with different substrates of OsCAS–YFP, the K_m for potassium cyanide was 0.67 mM and the K_m for cysteine was 1.07 mM, as revealed by double-reciprocal plots (Fig. 5).

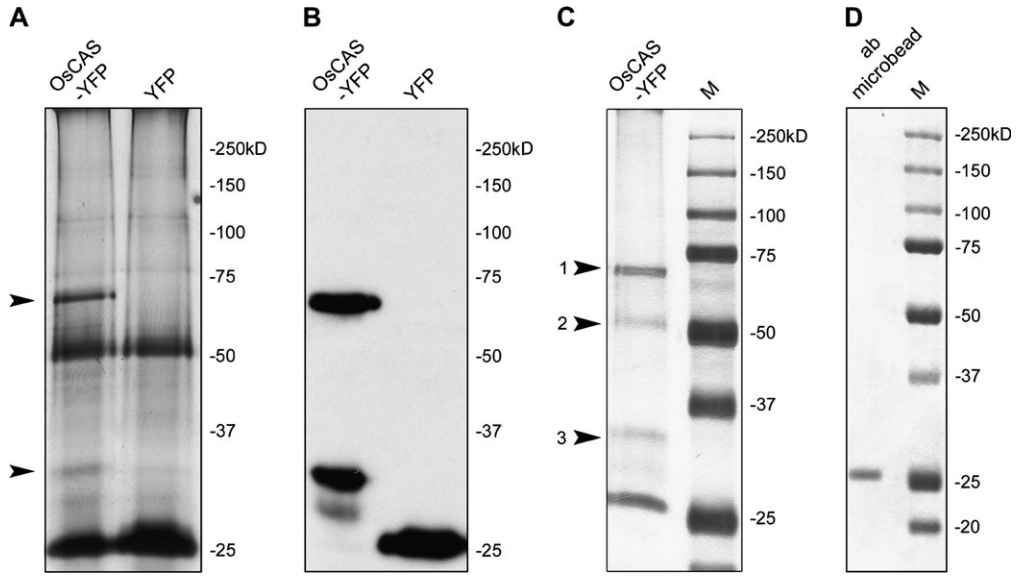

Fig. 4. Detection and identification of immuno-pull-down proteins from OsCAS–YFP and YFP transgenic lines. Immuno-pull-down proteins from these two transgenic lines washed with low stringency buffer were applied for silver staining (A) and immuno-detection of YFP-tagged protein, respectively (B). Arrows point out the differences in patterns of immuno-pull-down proteins between those lines in (A). To further reduce the background of immuno-pull-down proteins, immuno-pull-down proteins from OsCAS–YFP lines washed with high stringency buffer supplied in the kit were visualized by Coomassie blue staining. Four immuno-pull-down proteins were detected and identified (C, labelled with numbers). In a negative control, immobilized anti-GFP antibody was applied for Coomassie blue staining (D).

Table 1. OASS/CAS activities and kinetic parameters of OsCAS in rice, OsCAS–YFP in transgenic *Arabidopsis*, and OsCAS–YFP yeast transformant

K_m values were calculated by linear regression of double-reciprocal plots. Measurements of enzyme activities and determinations of K_m values were repeated at least three times.

Organisms	OASS-specific activity (μmol Cys min^{-1} mg^{-1} protein)	CAS-specific activity (μmol H_2S min^{-1} mg^{-1} protein)	Activity ratio of CAS/OASS	K_m for CAS reaction (mM)		Inhibition on CAS activity
				Cys	KCN	Cyanide IC_{50} (mM)[a]
Rice	N.D.[b]	13.3[c]	/	0.84	0.27	/
Arabidopsis	0.92±0.06	181±13.89	197	1.07±0.20	0.67±0.15	20
Yeast	1.84±0.77	111.3±5.11	60.5	0.91±0.11	0.31±0.01	40

[a] The concentration of cyanide required for 50% inhibition of CAS activity. IC_{50} was determined as 20 mM for spinach CAS previously by Warrilow and Hawkesford (2000).
[b] N.D., Not detected.
[c] CAS specific activity was determined on partially purified fraction (750-fold) from etiolated rice seedling extract.

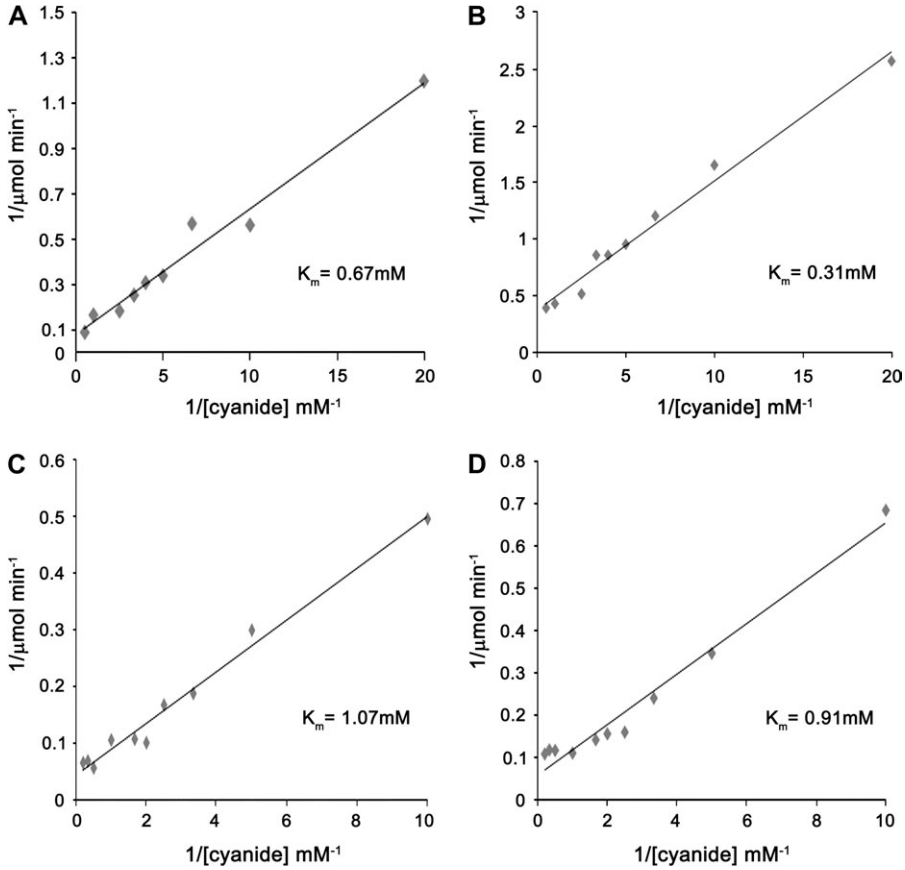

Fig. 5. Double reciprocal plots for determining K_m values of cyanide (A, B) and cytsteine (C, D) on heterologous OsCAS. Kinetic assays were conducted on OsCAS from transgenic *Arabidopsis* (A, C) and yeast (B, D).

Localization of OsCAS–YFP protein from OsCAS–YFP overexpression in Arabidopsis

To determine the transit peptide of OsCAS, a comparison was made between the N-terminal from OsCAS full-length encoding sequence (accession number AAV48542) and mature OsCAS from the transgenic line of which the N-terminal sequencing had been identified by a protein sequencer (Hewlett-Packard protein sequencer G1000A). The peptide sequence from 1 to 35 of OsCAS may serve as a transit peptide as mature OsCAS starts from peptide sequence 36 of the full-length encoding sequence. This transit peptide cleavage pattern of OsCAS (F↓STLQQ…) is classified as mitochondrial targeting sequence class II according to Zhang *et al.* (2001). This class is predominant, occupying 44.4% of all experimentally determined mitochondrial targeting sequences available (Zhang *et al.*, 2001). In addition, a transit peptide has been identified targeting mitochondria from the N-terminus of a full-length amino acid from OsCAS *in silico* by the two topology prediction programs iPSORT (Bannai *et al.*, 2002) (http://hc.ims.u-tokyo.ac.jp/iPSORT/) and MitoProt (Manuel and Claros, 1996) (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html); these results also suggest that OsCAS may be targeted to mitochondria *in silico*.

To determine the subcellular localization of OsCAS in transgenic *Arabidopsis*, root tip cells were prepared from germinating transgenic and YFP lines, followed by chemical fixation and preparation of ultra-thin sections for immunogold labelling with GFP antibodies. GFP antibodies labelled mitochondria predominantly (Fig. 6C), and these gold particles were evenly distributed in the mitochondrion matrix, although gold particles was also found in the cytoplasm (3 dots in the cytoplasm and 12 dots inside mitochondria in Fig. 6C). It is noted that these particles in the cytoplasm may indicate YFP instead of OsCAS–YFP as a start codon is located just upstream of the encoding sequence of the YFP gene in the binary construct pBA002 overexpressing OsCAS. From the control YFP line, anti-GFP antibody labelled cytoplasm in cells without any import to mitochondria (Fig. 6A, B). This demonstrated that OsCAS mainly targeted mitochondria.

Elucidation of the native form of OsCAS–YFP and some amino acid residues which are important for CAS activity

To elucidate the native form of OsCAS *in planta*, the native form of OsCAS–YFP was 'fixed' by incubating various concentrations of cross-linking agent BS^3 with extracts from OsCAS–YFP line 4 or the YFP transgenic line. After incubation with crude extracts from both transgenic lines and BS^3, proteins were separated in SDS–PAGE prior to visualization by Coomassie blue staining. YFP cross-reacting

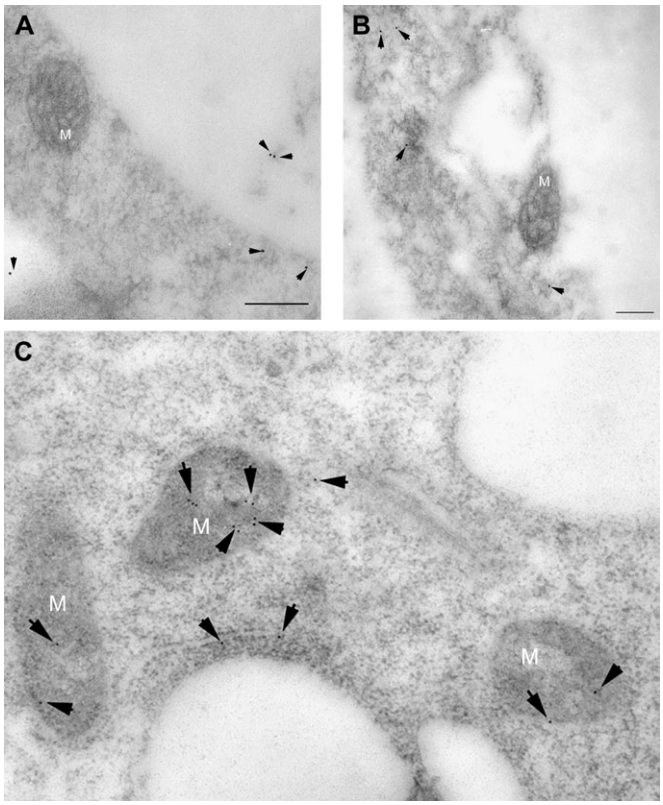

Fig. 6. Immunogold EM localizations of OsCAS fusion protein in root tip cells of the transgenic OsCAS–YFP line and its control YFP line. Immunogold particles were observed in cytoplasm in the control YFP line without signal in mitochondria (A, B) while those particles were confined in mitochondria predominantly in the transgenic OsCAS–YFP line (C). The black arrows indicate gold particles. M, Mitochondrion. Scale bar=200 nm.

bands were revealed by western blotting with the anti-GFP antibody. Molecular masses of protein bands were estimated by a software Quantity One version 4.6.1 from Bio-Rad Laboratories. In the western blotting, an anti-GFP cross-reacting band with a molecular mass about 64 kDa appeared in the OsCAS line 4 when no cross-linker was applied (Fig. 7A, upper panel). Following an increase in concentrations of the cross-linking reagent, this anti-GFP cross-reacting band shifted from 64 kDa to about double its original mass of roughly 130 kDa; and the intensity of this 130 kDa band increased following the increase of cross-linker BS^3 from 1 mM to 2 mM. This new band was found in OsCAS–YFP extracts with BS^3 but not in YFP extracts with BS^3. As several known signals were detected in the OsCAS transgenic line and the control *Arabidopsis* lines, a chemical cross-linking experiment with different concentrations of BS^3 from the OsCAS–HIS (consecutive histidine residues) overexpressing yeast was conducted (Fig. 7B, upper panel). A shift in anti-HIS cross-reacting band with a molecular mass of ~40–80 kDa followed the gradual increase of the concentration in BS^3. It is noted that several unknown signals were cross-reacted with anti-GFP antibody, but not anti-HIS antibody, hence these bands may be raised by non-specificity of anti-GFP antibody. As a ratio between the OsCAS complex form with either YFP or HIS_6 peptide and its monomer was close to 2, this indicates that the native OsCAS is possibly in a dimeric form.

As the native form of OsCAS is in dimeric form, monomers in an OsCAS dimer may work independently for CAS activity. To investigate the feasibility that all catalytic domains for CAS activity come from an individual monomer, both functional OsCAS and non-functional OsCAS were overexpressed in yeast. For a non-functional OsCAS, a mutant OsCAS K99G–HIS was made in which critical lysine for PLP binding was mutated. CAS activity of pure OsCAS–YFP from yeast was 111.3 ± 5.11 μmol H_2S min^{-1} mg $protein^{-1}$; no CAS activity was detected from OsCAS K99G–HIS (Fig. 8). While there was only one CAS activity band in yeast transformed with pYES2 OsCAS–YFP (Fig. 9A, lane 1), there were two CAS activity bands in yeast co-transformed with pYES2 OsCAS–YFP and pYES3 OsCAS K99G–HIS (Fig. 9A, lane 2). If the upper activity band in this lane was from homodimeric OsCAS–YFP in yeast transformed with pYES2 OsCAS–YFP, the lower activity band should come from the heterodimer made from OsCAS–YFP and OsCAS K99G–HIS in the co-transformed yeast. To address whether this activity band came from the heterodimer, this complex was purified by a nickel column and then each fraction in the purification was subjected to CAS in-gel assay. This complex was purified successfully (Fig. 9B, lane 7) and was recognized by both anti-HIS antibody and anti-GFP antibody (Fig. 9C). The western blotting strongly suggested that this complex is a heterodimer, consisting of a wild-type OsCAS–YFP and a mutant OsCAS K99G–HIS. As all domains for CAS activity are confirmed from an individual monomer, it can be concluded that dimer formation as well as PLP binding are essential for CAS activity.

Discussion

Auxin and ethylene regulate the expression of *OsCAS* transcripts in an antagonistic manner. Auxin serves to up-regulate *OsCAS* while ethylene serves to down-regulate *OsCAS* in etiolated rice seedlings. The promotion effect of auxins is much stronger than the suppression effect of auxin-induced ethylene (Kang *et al.*, 1971; Yu *et al.*, 1979) on *OsCAS* transcription. Etiolated monocot leaves possess a greater capability of ACC accumulation and thus ethylene production than dicot plants in their seedling stages (Jiao *et al.*, 1987). This antagonistic regulation may have evolved in monocot plants and be crucial for fine-tuning the *OsCAS* expressions to metabolize the cyanide formed concurrently with high ethylene biosynthetic rates. Several previous reports from dicots showed that exogenous ethylene would promote CAS gene transcription and thus CAS activity. Auxinic herbicides, such as 2,4-D and quinclorac (Grossmann and Kwiatkowski, 1995), are known to be effective in killing dicot weeds in grain fields. It is possible that the difference in transcription regulation of CAS genes between monocot and dicot may involve this phenomenon.

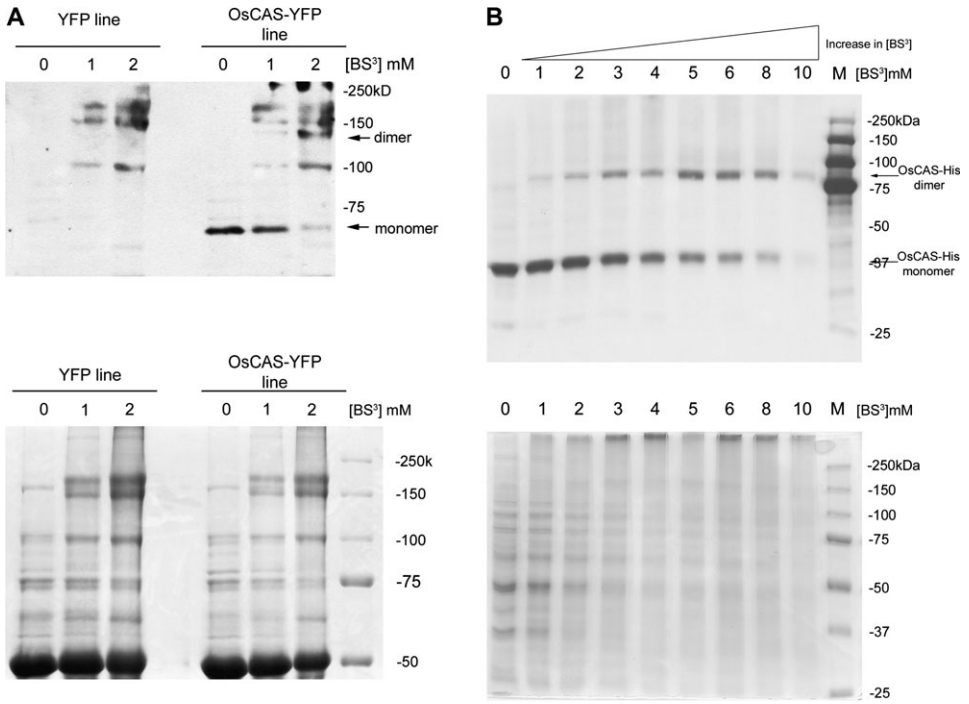

Fig. 7. Immunodetection of YFP-tagged protein from transgenic lines and yeast transformants incubated with chemical cross-linker BS^3. YFP tag or HIS_6 tag fused with OsCAS from either 40 μg of crude extracts from the transgenic line (A) or 30 μg of crude protein from the yeast transformant (B), respectively, incubated with various concentrations of BS^3, were immune-detected with anti-GFP (A) antibody or anti-HIS antibody (B). The arrows indicate monomeric and dimeric forms of OsCAS–YFP (upper panel). Equal loading of crude extracts were visualized by Coomassie blue staining (lower panel).

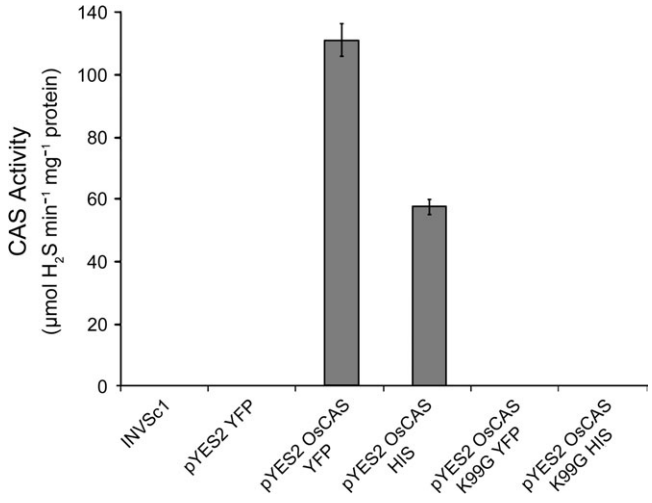

Fig. 8. CAS activities of various purified OsCAS mutants from heterologous expression in yeast strain INVSc1 were determined.

Enzymology studies on recombinant CAS expressed in bacterial mutant NK3 strains including potato (Maruyama *et al.*, 2001), *Arabidopsis*, and spinach (Hatzfeld *et al.*, 2000) have been reported. It is noted that there is a significant difference in the kinetic parameters of spinach CAS by comparing the native CAS with its recombinant protein (Ikegami *et al.*, 1988; Hatzfeld *et al.*, 2000). This is the first report on heterologous CAS expression in plants and in yeast. It has not been possible to immuno-purify OsCAS from rice seedlings or transgenic *Arabidopsis* by the anti-peptide anti-serum raised against a specific peptide in OsCAS; however, the purification of the recombinant OsCAS from transgenic *Arabidopsis* lines has been successful using an immobilized GFP antibody column. It is important to note that a shorter tag, like HIS_6 or MYC polypeptide, may hamper affinity of substrate binding of OsCAS instead of YFP protein (K_m for cyanide on OsCAS with either HIS_6 or MYC polypeptide almost increased at least ~8-fold compared with values from OsCAS from seedlings or OsCAS–YFP; KW Lai and KM Kwan, unpublished results) the same as OASS (Wirtz and Hell, 2006); thus, YFP protein fused at the C-terminal of OsCAS facilitates purification with an immunoaffinity-column. In addition, significant reduction in CAS activity of OsCAS in a relatively high concentration of cyanide is in agreement with Warrilow and Hawkesford (2000) on the cyanide inhibitory effect on CAS activity of CAS members.

Visualization of OsCAS in root tip mitochondria by electron microscopy on immunogold labelling with GFP antibodies is in agreement with previous reports which identified subcellular localization of CAS activity by organelle fractionation methods (Akopyan *et al.*, 1975; Wurtele *et al.*, 1985). However, in those previous studies CAS activity in the mitochondrial fraction might have originated from mitochondrial OASS or other OASSs due to impure preparation. Hence, the present result is the first to reveal the mitochondrial localization of OsCAS in plant cells and at the same time provide evidence for its role in cyanide detoxification.

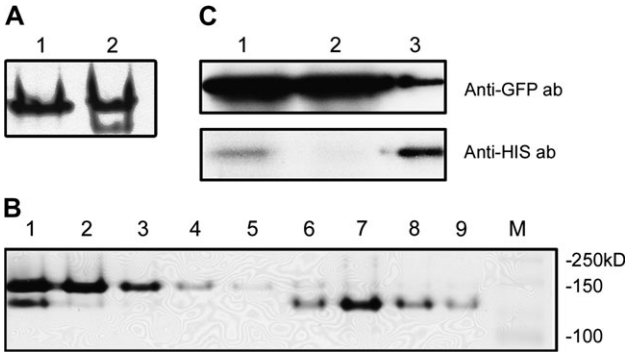

Fig. 9. In-gel CAS activity assay and immunodetection on wild-type OsCAS–YFP homodimer and OsCAS–YFP/OsCAS K99G–HIS heterodimer. In-gel CAS activities on 20 μg of crude proteins from yeast transformants with wild-type OsCAS–YFP (A, lane 1) and co-expressions of OsCAS–YFP/OsCAS K99G–HIS (A, lane 2) were determined. In-gel CAS activity assay on fractions during the purification of the heterodimer through the nickel column from the yeast transformant with co-expressions of OsCAS–YFP/OsCAS K99G–HIS (B) were conducted. Twenty micrograms of fractions from crude extract (lane 1), flow through (lane 2), three washing steps (lanes 3–5), and four elution steps (lanes 6–9) were applied in the assay. Immunodetection of YFP- or HIS-tagged protein on fractions from crude (C, lane 1), flow through (C, lane 2), and purified heterodimer (C, lane 3) were detected by anti-GFP antibody and anti-HIS antibody, respectively.

By combining results on the homodimeric native form from chemical cross-linking on OsCAS protein from transgenic lines and 35 amino acid residues from the N-terminal of the full-length OsCAS cleaved from mature OsCAS protein in those lines by Edman degradation, the molecular mass of OsCAS is calculated as 73 kDa. This 35 residue cleavage polypeptide may serve as a mitochondrial signal peptide facilitating targeting of OsCAS.

A protein model of *Arabidopsis* cytosolic OASS, which shares a similar amino acid sequence, suggests that a monomer consists of all critical sites and a PLP for facilitating its function without sharing these sites; however, native OsCAS or CAS family in other plant species are reported to be homodimeric in form. The importance of dimerization to its functions has not been addressed. CAS activity was detected from a dimer composed of a wild-type OsCAS monomer and an OsCAS monomer mutated at the PLP-binding lysine residue. Hence, the present results showed that one intact monomer with PLP cofactor binding within a dimer would be sufficient for CAS activity. It is speculated that dimerization may provide the necessary conformational change(s) on OsCAS monomer for its catalytic activity.

Protein–protein interaction of plant OASS to SAT has been reported in early purification of the cysteine synthase complex from plants (Droux *et al.*, 1992; Nakamura *et al.*, 1987), a complex association in co-expression of *Arabidopsis* OASS/SAT in a bacterial system (Droux *et al.*, 1998), and a yeast two-hybrid system in screening interactions between proteins encoding the tobacco expression library and bacterial OASS/SAT (Liszewska *et al.*, 2005). Association/dissociation of this complex has been widely accepted as a regulation mechanism for cysteine synthesis in plants (Droux *et al.*, 1992; Wirtz *et al.*, 2001) and this interaction is generally accepted as a criterion for an authentic OASS (Jost *et al.*, 2000). Because of the highly conserved amino acid sequence of OsCAS to those OASS, one may suspect that OsCAS may play dual roles in cyanide metabolism and cysteine synthesis by recycling sulphide liberated from the CAS reaction inside mitochondria, as there is no other rice OASS/CAS member predicted in mitochondrial *in silico*. This prompted us to conduct a simple experiment regarding the interaction of OsCAS to watermelon (Wt) SAT in yeast as this interaction is highly plastic among species (Droux *et al.*, 1998; Liszewska *et al.*, 2005). The results (Fig. 10) showed that OsCAS could not interact with WtSAT in yeast despite the interaction of SpOASS to WtSAT being demonstrated. In addition, there was no protein co-precipitated with OsCAS from transgenic *Arabidopsis* lines; these two lines of evidence might indicate that OsCAS could not interact with SAT *in planta* or in yeast. These findings agree well with the work of Bonner *et al.* (2005) on SAT interaction with AtOASS in *Arabidopsis*. The three critical amino acid residues facilitating SAT interaction on AtOASS have been examined and those amino acid residues in OsCAS and other reported CAS compared and it was discovered that these three amino acid residues are not fully conserved in those of OsCAS and in most of the CAS reported from other species. The present results thereby strongly suggested that OsCAS play a role in cyanide detoxification but are not involved in sulphide recycling inside the mitochondria.

Contrary to CAS activity which was mostly found in mitochondria, cysteine synthesis have been predicted in different subcellular compartments including cytoplasm, chloroplasts, and mitochondria, each compartment using their own allocation of OASS and SAT to make the cysteine synthase complex. OASS and SAT are thereby encoded by two gene families and members are targeted by different compartments (Wirtz and Hell, 2006). Based on the results of the present search, the rice genome contains nine members in the OASS/CAS gene family. *OsCAS* is the sole sequence encoding CAS and the sole sequence encoding protein predicted (or proven in this study) to be localized to mitochondria. Also it was not possible to find a putative gene coding for mitochondrial SAT in the rice genome database search. This raises the question as to how cysteine can be synthesized in rice mitochondria—recycled from hydrogen sulphide as a result of CAS action—and whether cysteine can be imported to mitochondria from the cytoplasm in rice. A recent publication of Watanabe *et al.* (2008) reported the evidence on intracellular cysteine transport between cytosol, mitochondrion, and plastid in *Arabidopsis* OASS T-DNA knockout lines. Their report suggested that the cytosolic OASS would be more critical in cysteine synthesis than other OASS in other compartments. In rice, it is logical to propose that maintaining the mitochondrial cysteine pool while the ethylene production rate is high may depend on cysteine import from the

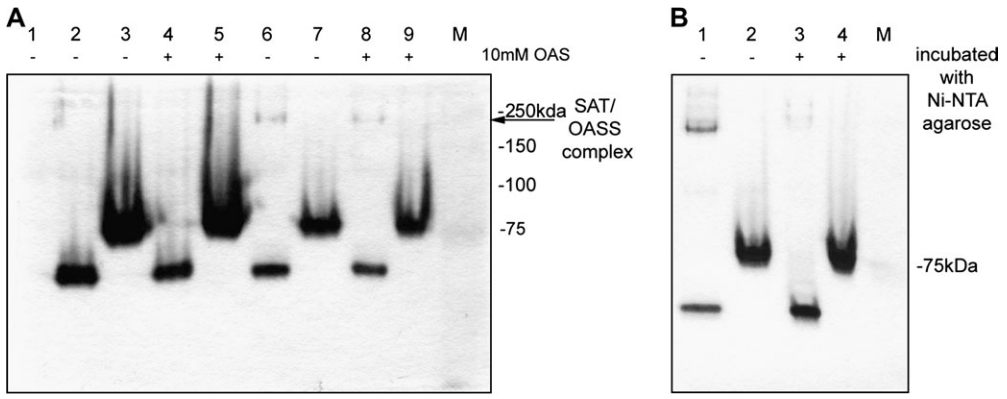

Fig. 10. Detection of a cysteine synthase complex in yeasts with co-expressions of SpOASS or OsCAS with WtSAT. In-gel CAS activity assay (A) was conducted on 100 μg crude protein from yeast transformants with pYES2 HIS–WtSAT (lane 1), pYES3 SpOASS–MYC (lanes 2 and 4), pYES3 OsCAS–MYC (lanes 3 and 5), co-expression of pYES2 HIS–WtSAT and pYES3 SpOASS–MYC (lanes 6 and 8), and co-expression of pYES2 HIS–WtSAT and pYES3 OsCAS–MYC (lanes 7 and 9). A '+' sign represents an addition of OAS prior to loading and an arrow indicates a putative cysteine synthase complex. To demonstrate further that SAT is a component in this putative cysteine synthase complex, an in-gel CAS activity assay (B) was conducted on 100 μg crude protein (lanes 1 and 2) and protein pre-incubated with nickel agarose (lanes 3 and 4) from yeast transformant with co-expression of pYES2 HIS–WtSAT and pYES3 SpOASS–MYC (lanes 1 and 3)or co-expression of pYES2 HIS–WtSAT and pYES3 OsCAS–MYC (lanes 2 and 4). Pre-incubation extracts from yeast co-expressing SpOASS and WtSAT with nickel agarose would remove this additional CAS-stained band (lanes 1 and 3) and this demonstrated the interaction between SpOASS to WtSAT. SAT-specific activities from yeast transformants with co-expression of pYES2 HIS–WtSAT and either pYES3 SpOASS–MYC or pYES3 OsCAS–MYC were 86.32 μmol 5-thio-2-nitrobenzoate min^{-1} mg^{-1} protein and 74.00 μmol 5-thio-2-nitrobenzoate min^{-1} mg^{-1} protein, respectively, following the spectrophotometric assay from Bonner *et al.* (2005).

cytosol. The likely mechanism is that hydrogen sulphide liberated from the CAS reaction is either transported or diffused from mitochondria to cytsoplasm (Wirtz and Hell, 2007) in which cysteine synthesis may take place and then be transported back to the mitochondria to sustain cyanide detoxification. However, identification of the mitochondrial cysteine permease or the import mechanism of cysteine to mitochondria has not been fully elucidated.

Supplementary data

Results regarding integrations of OsCAS–YFP and YFP encoding sequences in the *Arabidopsis* genome by Southern blotting (Fig. S1) and results of the protein identities for immuno-purified proteins by database searching (Table S1) have been supplemented and this material is available at *JXB* online.

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