

# The *Arabidopsis* DJ-1a protein confers stress protection through cytosolic SOD activation

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## Summary

Mutations in the *DJ-1* gene (also known as *PARK7*) cause inherited Parkinson's disease, which is characterized by neuronal death. Although DJ-1 is thought to be an antioxidant protein, the underlying mechanism by which loss of DJ-1 function contributes to cell death is unclear. Human DJ-1 and its *Arabidopsis thaliana* homologue, AtDJ-1a, are evolutionarily conserved proteins, indicating a universal function. To gain further knowledge of the molecular features associated with DJ-1 dysfunction, we have characterized AtDJ-1a. We show that AtDJ-1a levels are responsive to stress treatment and that AtDJ-1a loss of function results in accelerated cell death in aging plants. By contrast, transgenic plants with elevated AtDJ-1a levels have increased protection against environmental stress conditions, such as strong light, H<sub>2</sub>O<sub>2</sub>, methyl viologen and copper sulfate. We further identify superoxide dismutase 1 (SOD1) and glutathione peroxidase 2 (GPX2) as interaction partners of both AtDJ-1a and human DJ-1, and show that this interaction results in AtDJ-1a- and DJ-1-mediated cytosolic SOD1 activation in a copper-dependent fashion. Our data have highlighted a conserved molecular mechanism for DJ-1 and revealed a new protein player in the oxidative stress response of plants.

**Key words:** Arabidopsis, Stress, DJ-1

## Introduction

Parkinson's disease (PD) is a multifaceted chronic neurodegenerative disease characterized by early and extensive loss of dopaminergic neurons in the pars compacta of the substantia nigra. PD etiology remains obscure and appears sporadic in nature in most instances. However, genetic lesions have been associated with early- and late-onset PD (Bonifati et al., 2003; Kitada et al., 1998; Paisan-Ruiz et al., 2004; Polymeropoulos et al., 1997; Valente et al., 2004; Zimprich et al., 2004). Several genes have been linked to familial PD, including  $\alpha$ -synuclein (Polymeropoulos et al., 1997), Parkin (Kitada et al., 1998), PINK1 (Valente et al., 2004) and DJ-1 (also known as PARK7) (Bonifati et al., 2003). Mutations in DJ-1/PARK7 cause early-onset Parkinsonism with abnormalities in the dopaminergic system resembling those of sporadic PD (Bonifati et al., 2003; van Duijn et al., 2001). DJ-1/PARK7 is predominantly cytosolic, ubiquitously expressed in both brain and peripheral tissue (Zhang et al., 2005), and involved in oncogenesis (Nagakubo et al., 1997), neuroprotection (Kim et al., 2005; Park et al., 2005), male fertility (Okada et al., 2002), control of protein-RNA interactions (van der Brug et al., 2008) and modulation of androgen receptor transcription activity (Takahashi et al., 2001; Tillman et al., 2007). DJ-1/PARK7 also has protease and chaperone activities (Olzmann et al., 2004; Shendelman et al., 2004). Evidence has demonstrated that DJ-1/PARK7 is responsive to oxidative stress, indicating a protection role against harsh conditions (Canet-Aviles et al., 2004; Gu et al., 2009; Kim et al., 2005; Martinat et al., 2004; Meulener et al., 2006); however, its precise mode of action remains unknown.

Reactive oxygen species (ROS) are important products of aerobic metabolism in cells. Commonly, ROS have been viewed as toxic superoxide by-products; however, they also behave as

important regulators of growth and development (Apel and Hirt, 2004; Miller et al., 2009). In plants, ROS act as signaling molecules to control processes such as programmed cell death, the cell cycle, hormone signaling, cell elongation, and responses to abiotic and biotic stress conditions (Apel and Hirt, 2004). Indeed, studies have shown that ROS generated by NADPH oxidase control plant-cell expansion through activation of calcium channels (Foreman et al., 2003).

Superoxide radicals represent one of the main ROS during cellular oxidative stress responses. Plants, because of their sessile nature, have developed effective defense systems against superoxide radicals. This includes limiting ROS generation as part of normal cell differentiation and development, as well as removal of ROS during periods of excessive stress. This balanced fine-tuning is controlled by superoxide dismutase (SOD)/copper superoxide dismutase 1 (CSD1) (Alscher et al., 2002). Several studies have convincingly shown that various stress conditions, such as UV irradiation, excess light and methyl viologen, modulate SOD transcript and enzyme activity levels (Gomez et al., 1999; Kliebenstein et al., 1998); however, the exact mechanisms controlling SOD activity in plants are still unclear (Van Camp et al., 1997).

SOD catalyzes the dismutation of superoxide and therefore constitutes the first line of defense against ROS (Bowler et al., 1991). SOD has clear physiological importance; dysfunctional SOD in *Drosophila* and mice leads to a range of severe pathologies and early mortality (Elchuri et al., 2005; Li et al., 1995; Phillips et al., 1989). In humans, inappropriate SOD activity can result in motor neuron disease, such as amyotrophic lateral sclerosis (ALS) (Deng et al., 1993), and altered SOD activity has been associated with numerous pathologies (Noor et al., 2002). In plants, SOD

inactivation leads to severe phenotypes, including growth retardation, photooxidative damage, DNA damage and lipid peroxidation (Alscher et al., 2002)

A direct molecular link between oxidative stress protection, DJ-1 and SODs has not been reported. However, it has been shown that, in NIH3T3 mouse cell lines, DJ-1 loss of function results in decreased levels of extracellular *SOD3* transcript, whereas transcript levels of other SODs are unchanged (Nishinaga et al., 2005). To address the relationship between DJ-1 and SOD at the molecular level, we have characterized the *Arabidopsis* DJ-1 homolog, AtDJ-1a, and show that DJ-1 and SOD act in concert at the protein level through an evolutionarily conserved mechanism.

## Results

### *Arabidopsis thaliana* harbors DJ-1/PARK7 homologs

Using the human DJ-1/PARK7 amino acid sequence as an input query, we identified three DJ-1/PARK7 homologs, AtDJ-1a (At3g14990), AtDJ-1b (At1g53280) and AtDJ-1c (At4g34020), in the *Arabidopsis* nuclear genome, showing 35%, 41% and 35% similarity to human DJ-1/PARK7, respectively (supplementary material Fig. S1). Amino acid comparisons indicate a gene duplication event, whereby two complete DJ-1/PARK7 polypeptides are present in AtDJ-1a, AtDJ-1b and AtDJ-1c (Fig. 1A; supplementary material Fig. S1). All three AtDJ-1 homologs contain functionally conserved DJ-1/PARK7 amino acid residues, including residues associated with PD conditions (Fig. 1A; supplementary material Fig. S1). To address the subcellular localization of the AtDJ-1 homologs, we expressed AtDJ-1a-, AtDJ-1b- and AtDJ-1c-YFP fusion proteins in transgenic *Arabidopsis* plants. We found that AtDJ-1b and AtDJ-1c are targeted to plastids (supplementary material Fig. S2), whereas AtDJ-1a localizes to the cytosol and nucleus (Fig. 1B-D), as observed for DJ-1/PARK7 in CHO-S cells (Fig. 1E) (Shendelman et al., 2004; Shinbo et al., 2005). Reports suggest that DJ-1/PARK7 also translocates to mitochondria in response to C106 oxidation (Canet-Aviles et al., 2004); however, we observed only isolated cases of mitochondria-

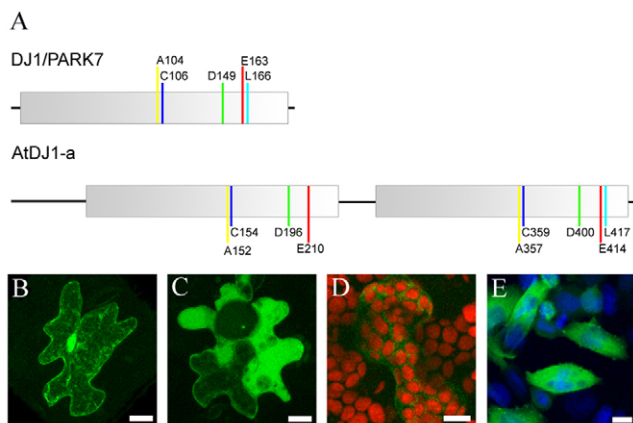
targeted AtDJ-1a in *Arabidopsis* (data not shown). It is possible that the presence of three DJ-1 homologs in plants alleviates the absolute necessity for mitochondrial-specific translocation of AtDJ-1a. Because AtDJ-1a has similar localization patterns to DJ-1/PARK7, AtDJ-1a was selected for further study.

### AtDJ-1a expression is stress induced

DJ1/PARK7 is involved in the oxidative stress response directly affecting cell death (Sekito et al., 2006; Taira et al., 2004) and *Arabidopsis* transcripts encoding proteins involved in stress pathways are often upregulated in response to external stress cues. To test whether AtDJ-1a responds to stress treatment, we cloned the 1358-nucleotide genomic promoter region of *AtDJ-1a* upstream of the  $\beta$ -glucuronidase (*GUS*) reporter gene (AtDJ-1pro/*GUS*) and generated AtDJ-1pro/*GUS*-expressing transgenic *Arabidopsis* plants. *GUS* activity assays revealed that AtDJ-1a is expressed throughout young seedlings, but becomes more confined to vascular tissue in older plants (Fig. 2A,D). We further examined the possible upregulation of *AtDJ-1a* in response to stress treatment, including strong light (280  $\mu\text{mol}/\text{m}^2/\text{second}$ ) (Fig. 2B),  $\text{CuSO}_4$  (Fig. 2C),  $\text{H}_2\text{O}_2$  (Fig. 2E) and methyl viologen (MV) (Fig. 2F). Transgenic AtDJ-1pro/*GUS* plants showed dramatic *AtDJ-1a* upregulation in response to all stress treatments applied, as demonstrated by increased *GUS* activity throughout the leaves (Fig. 2B). These results demonstrate that transcriptional control of *AtDJ-1a* is regulated by a spectrum of exogenous stress cues, including treatments that induce oxidative stress.

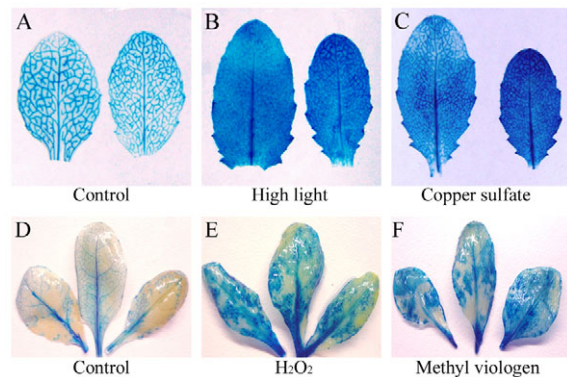
### Loss-of-function AtDJ-1a causes cell death, whereas elevated AtDJ-1a levels have a protective effect in *Arabidopsis*

Mutations within *DJ-1/PARK7* lead to apoptosis through increased susceptibility to cell-death agents such as  $\text{H}_2\text{O}_2$  (Canet-Aviles et al., 2004; Junn et al., 2005; Takahashi-Niki et al., 2004). To examine the effect of AtDJ-1a loss of function in *Arabidopsis*, we identified and characterized two T-DNA mutant lines [N830498 (Fig. 3A) and N551124]. Segregation and transcript analysis revealed N830498 (Fig. 3C,D) and N551124 (data not shown) to be AtDJ-1a recessive null mutants; the mutant alleles showed identical phenotypes. We performed a detailed characterization of



**Fig. 1. Domain structure of AtDJ-1a and its subcellular localization.**

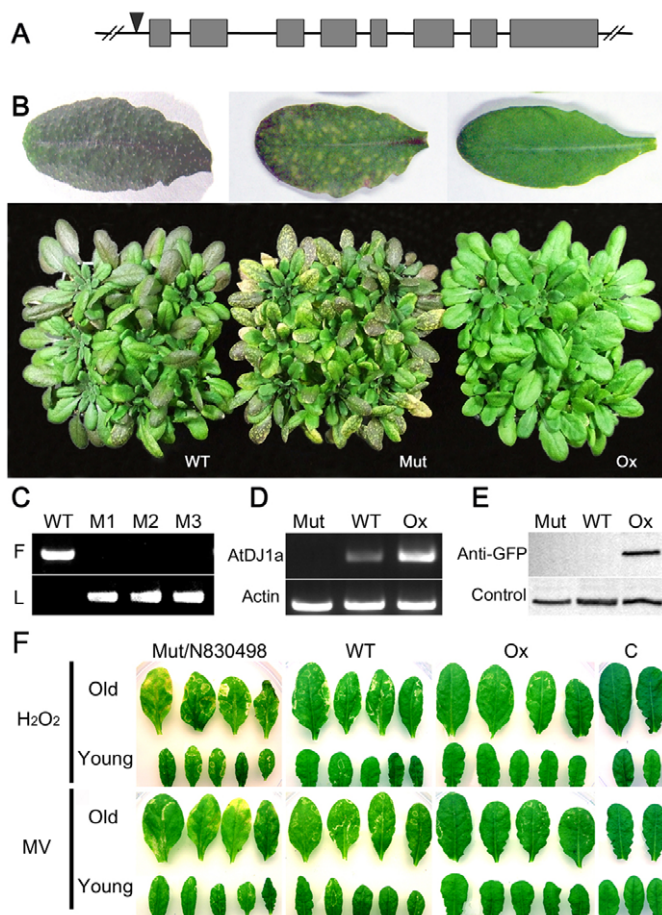
(A) Domain structure of AtDJ-1a and DJ1/PARK7, showing predicted DJ1 domains and conserved amino acid residues associated with PD. (B-D) Confocal images of 35S-AtDJ1a-YFP transgenic *Arabidopsis* plants showing AtDJ1a-YFP localization to the cytosol and nucleus in epithelial cells (B,C) and mesophyll cells (D). Red fluorescence in D represents chlorophyll. (E) Confocal image of CHO-S cells expressing DJ1/PARK7-GFP (green) stained with DAPI (blue). Scale bars: 10  $\mu\text{m}$



**Fig. 2. Expression analysis of AtDJ-1a in response to stress treatment.**

Histochemical analyses of *GUS* activity in 15-day-old transgenic AtDJ1a-GUS transgenic *Arabidopsis* plants grown under (A,D) standard conditions (control), (B) treated with elevated light (280  $\mu\text{mol}/\text{m}^2/\text{second}$ ), (C) treated with 1 mM  $\text{CuSO}_4$ , (E) treated with  $\text{H}_2\text{O}_2$  and (F) treated with MV.

the N830498 mutant; however, no growth or developmental phenotypes were observed under normal growth conditions (data not shown). Following this, we exposed wild-type and homozygous N830498 plants to identical elevated light irradiation ( $280 \mu\text{mol}/\text{m}^2/\text{second}$ ), as for the expression analysis experiments (Fig. 2B). In young rosette leaf stage seedlings (2 weeks old), no phenotypic consequences of AtDJ-1a loss of function were observed. However, in aging plants, once the inflorescence emerged (4 weeks old), leaves from N830498 mutant plants showed the presence of large, multiple lesions (Fig. 3B, mut), compared with a few small lesions in wild-type plants (Fig. 3B, WT), indicative of increased susceptibility to excess light stress and subsequent cell death. To test whether treatments that induce oxidative stress had a similar effect on AtDJ-1a loss-of-function mutants, we



**Fig. 3. AtDJ-1a deficiency results in cell-death lesions, whereas elevated levels protect *Arabidopsis* against oxidative stress.** (A) AtDJ1a genetic map showing the SALK N830498 T-DNA mutant insertion site 163 nucleotides upstream of AUG. (B) Phenotypes of wild-type (WT), mutant (Mut) and AtDJ1a-YFP-overexpressing (Ox) transgenic plants grown under elevated light conditions ( $280 \mu\text{mol}/\text{m}^2/\text{second}$ ). (C) Genetic analysis of homozygous plants (M1-M3) by PCR using gene-specific primers (F), a T-DNA-specific left board primer and a gene-specific primer (L). (D) Reverse transcriptase (RT)-PCR to confirm lack of *AtDJ-1a* transcript in N830498 and elevated *AtDJ-1a* transcript in transgenic *Arabidopsis* overexpressing an *AtDJ1a-YFP* transgene. (E) Western blot showing elevated AtDJ1a-YFP protein levels in transgenic *Arabidopsis* overexpressing an *AtDJ1a-YFP* transgene. (F) Phenotypic analysis of WT, mutant (Mut/N830498) and AtDJ-1a-overexpressing (Ox) transgenic plants in response to  $\text{H}_2\text{O}_2$  and MV treatment.

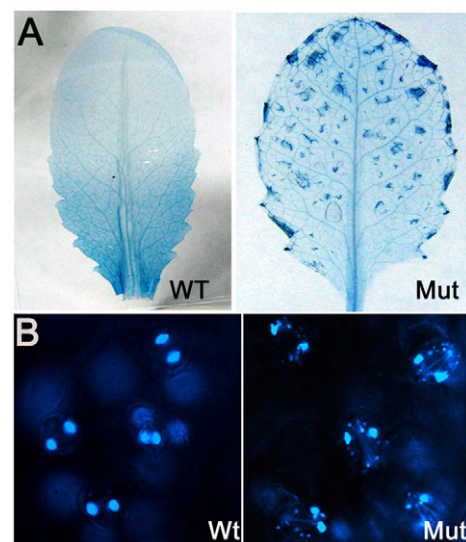
subjected wild-type and N830498 mutant plants to  $\text{H}_2\text{O}_2$  and MV. Plants were grown for three weeks and sprayed with a  $10 \mu\text{M}$  solution of  $\text{H}_2\text{O}_2$  or MV every 24 hours for one week. From these experiments, it was evident that both  $\text{H}_2\text{O}_2$  and MV induced multiple lesions in the N830498 mutant compared with few lesions in wild-type plants and that the effect was more pronounced in older tissue (Fig. 3F).

To confirm that the lesions were indeed due to a cell-death process, we performed trypan blue (Fig. 4A) and DAPI (Fig. 4B) staining. These experiments demonstrated clear cell death within the observed lesions, which exhibited pyknotic nuclei indicative of chromatin condensation, a hallmark of apoptotic cell death (Clarke, 1990). To confirm that no spurious genome mutations were the cause of the observed phenotype, we restored wild-type characteristics in N830498 by complementation with a *35S-AtDJ-1a/At3g14990* full-length cDNA (supplementary material Fig. S3). Transgenic plants with a similar level of *AtDJ-1a* transgene expression to *AtDJ-1a* expression levels in wild-type plants were analysed to eliminate effects due to AtDJ-1a overexpression.

The observed AtDJ-1a loss-of-function cell-death phenotype mirrors the neuronal cell death phenomenon in DJ-1/PARK7 mutant carriers (Uversky, 2004), suggesting that AtDJ-1a also has antioxidant properties in plants. To address the possible antioxidant protective role of AtDJ-1a, we generated 35S-AtDJ-1a gain-of-function transgenic *Arabidopsis* plants with elevated AtDJ-1a levels (Fig. 3D,E), which showed an absence of cell-death lesions in response to excess-light stress treatment (Fig. 3B, Ox) and in response to  $\text{H}_2\text{O}_2$  and MV (Fig. 3F, Ox). Combined, these data reveal that AtDJ-1a has a role in protecting plants in response to various stress conditions.

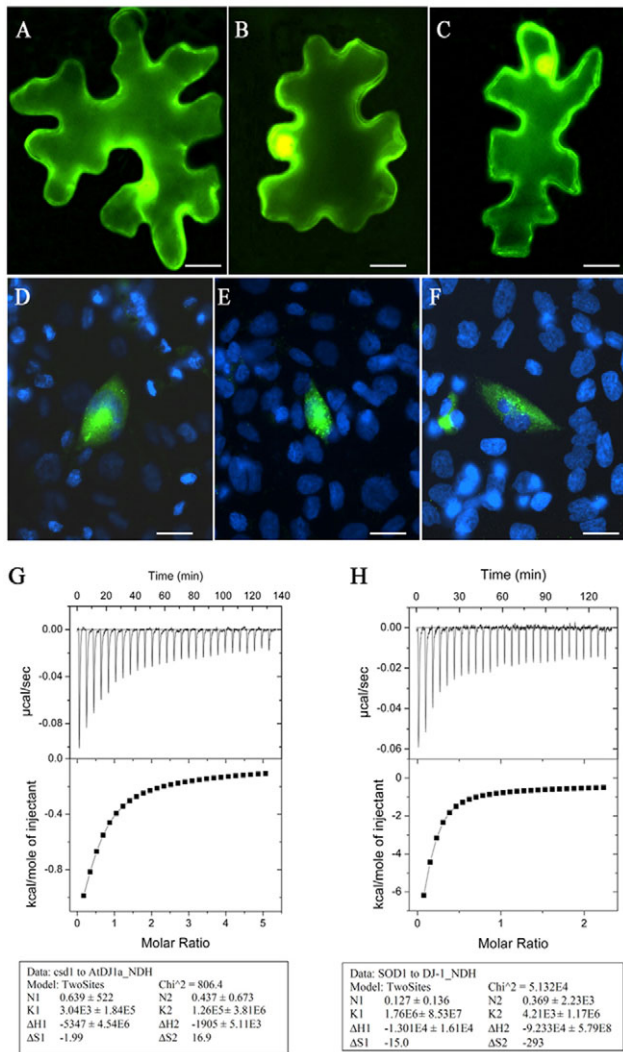
#### AtDJ-1a and DJ-1/PARK7 interact with CSD1 and SOD1

Several studies have demonstrated that the absence of DJ-1/PARK7 leads to increased susceptibility to  $\text{H}_2\text{O}_2$ -induced cell death (Kim et al., 2005; Martinat et al., 2004; Taira et al., 2004; Yokota et al.,



**Fig. 4. AtDJ-1a deficiency-induced lesions show cell-death characteristics.** (A) Trypan blue staining of WT and mutant (Mut) leaves showing cell-death lesions in response to loss of AtDJ-1a. (B) DAPI staining of WT and mutant (Mut) leaves showing enlarged and degrading nuclei in AtDJ-1a loss-of-function mutants.

2003). Because SOD catalyses the dismutation of damaging superoxide radicals to oxygen and  $H_2O_2$ , which in turn is converted into  $H_2O$  by glutathione peroxidase 2 (GPX2) (Raha and Robinson, 2000), we addressed whether AtDJ-1a has a functional interaction with these enzymes. We performed YFP bimolecular fluorescence complementation (BiFC) assays, showing that AtDJ-1a indeed interacts with CSD1 (Fig. 5A) and *Arabidopsis* GPX2 (AtGPX2) (Fig. 5B) in the plant cytosol and nucleus. Interestingly, we extended this analysis and showed that heterologously expressed DJ-1/PARK7 interacts with SOD1, the human homolog of *Arabidopsis* CSD1, when introduced into plant cells (Fig. 5C).



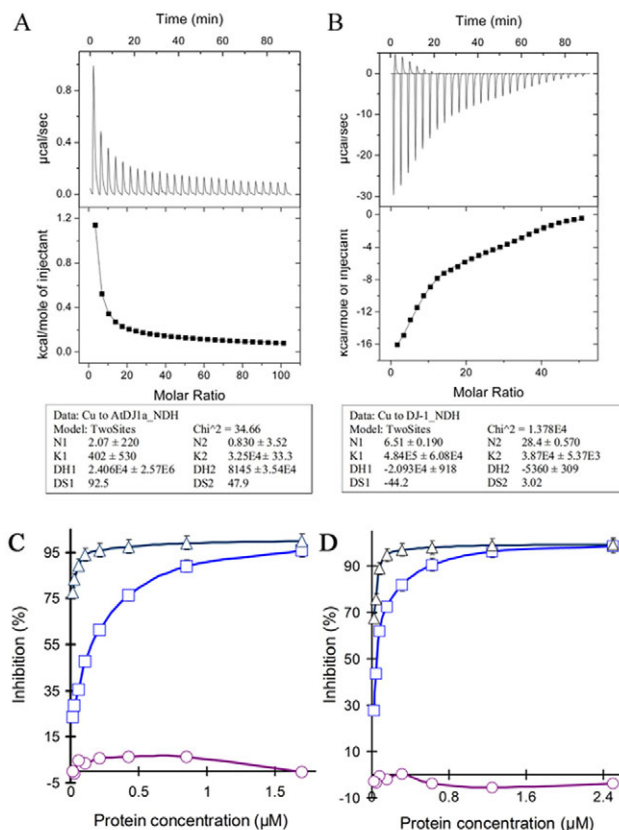
**Fig. 5. AtDJ-1a and DJ-1/PARK7 interact with CSD1 and SOD1, respectively, and AtGPX2 and GPX2, respectively.** (A–C) BiFC analysis in tobacco epithelial cells showing protein–protein interactions between (A) AtDJ-1a and CSD1, (B) AtDJ-1a and AtGPX2, and (C) DJ-1/PARK7 and SOD1 in the cytosol and nucleus. (D) BiFC analysis demonstrating dimerization of DJ-1/PARK7 in CHO-S cells. (E, F) BiFC analysis in CHO-S cells showing protein–protein interaction between (E) DJ-1/PARK7 and SOD1 and (F) DJ-1/PARK7 and GPX2 in the cytosol and nucleus. ITC analysis of (G) AtDJ-1a and CSD1, and (H) DJ-1/PARK7 and SOD1. In G, CSD1 was titrated against AtDJ-1a. In H, SOD1 was titrated against DJ-1/PARK7. In both cases, an increase in heat output can be observed, demonstrating protein–protein interactions. Scale bars: 10  $\mu$ m (A–F).

The interaction between DJ-1/PARK7 and SOD1 in plant cells prompted us to test interactions in a mammalian CHO-S cell line. As a control, we showed that DJ-1/PARK7 interacts with itself in BiFC assays in CHO-S cells, as previously reported (Fig. 5D) (Canet-Aviles et al., 2004). BiFC assays further demonstrated that DJ-1/PARK7 interacts with SOD1 (Fig. 5E; supplementary material Fig. S4) and GPX2 (Fig. 5F; supplementary material Fig. S4) in the cytosol of CHO-S cells. To further confirm the observed interaction between AtDJ-1a and DJ-1/PARK7 with CSD1 and SOD1, respectively, we performed isothermal calorimetric (ITC) assays. Purified CSD1 was titrated into a solution of purified AtDJ-1a, which resulted in an increase in temporal heat output, demonstrating direct interaction (Fig. 5G). Similarly, ITC analysis revealed that DJ-1/PARK7 titrated against SOD1 showed an increase in heat output (Fig. 5H), verifying the interaction between these two proteins. These findings demonstrate that AtDJ-1a and DJ-1/PARK7 interact with CSD1 and SOD1, respectively, and AtGPX2 and GPX2, respectively, in the cytosol and nucleus of plant and mammalian cells.

#### AtDJ-1a and DJ-1/PARK7 enhance the activity of CSD1 and SOD1

To address the functionality of the observed interactions, we measured CSD1 and SOD1 activity in response to interaction with AtDJ-1a and DJ-1/PARK7, respectively. As SOD activity is dependent on copper, we performed CSD1/SOD activity assays in the absence and presence of  $CuSO_4$ . As previously reported (Taira et al., 2004), copper-free DJ-1/PARK7 was unable to stimulate SOD1/CSD1 activity, as was copper-free AtDJ-1a (data not shown). Based on these findings, we tested whether AtDJ-1a and DJ-1/PARK7 are able to bind copper. AtDJ-1a and DJ-1/PARK7 were expressed and purified in the absence of copper, followed by ITC analysis, which clearly demonstrated binding of  $CuSO_4$  to both AtDJ-1a (Fig. 6A) and DJ-1/PARK7 (Fig. 6B; supplementary material Fig. S5). To test for the possible effect of copper-loaded AtDJ-1a and DJ-1/PARK7 on SOD stimulation, we expressed AtDJ-1a and DJ-1/PARK7 in the presence of  $CuSO_4$ , followed by affinity purification to remove any unbound copper. In contrast to copper-free AtDJ-1a and DJ-1/PARK7, copper-loaded AtDJ-1a and DJ-1/PARK7 stimulated CSD1 and SOD1, showing a marked increase in CSD1 (Fig. 6C) and SOD1 activity (Fig. 6D) in the presence of equimolar concentrations of copper-AtDJ-1a and copper-DJ-1/PARK7, respectively. As a control, we tested the effect of  $CuSO_4$  on the ITC assays and found no heat output due to the presence of  $CuSO_4$  in the buffer (supplementary material Fig. S5). Combined, these data suggest that AtDJ-1a and DJ-1/PARK7 might deliver copper to CSD1 and SOD1, leading to enzyme stimulation. To ensure that the lesion phenotype observed in AtDJ-1a loss-of-function (N830498 and N551124) mutants and the protective role observed in AtDJ-1a-overexpressing plants were not simply due to upregulation of CSD1, we performed semi-quantitative RT-PCR of CSD1 levels in the wild type, in AtDJ-1a-overexpressing plants and in the two AtDJ-1a loss-of-function mutants. From these studies, we found that CSD1 mRNA levels are unchanged in all plants (supplementary material Fig. S6).

We also tested the notion that AtDJ-1a and DJ-1/PARK7 might have an effect on AtGPX2 and GPX2 activity; however, no increase in glutathione peroxidase activity was detected (data not shown).



**Fig. 6. AtDJ-1a and DJ-1/PARK bind copper and mediate stimulation of CSD1 and SOD1 activity.** (A,B) ITC analysis showing that AtDJ-1a (A) and DJ-1/PARK7 (B) bind copper. (C,D) SOD enzyme assays based on the inhibition of xanthine oxidase (XO). (C) Stimulation of CSD1 activity by AtDJ-1a. CSD1 was serially diluted in the absence (□) or presence (Δ) of 2 μM AtDJ-1a. AtDJ-1a promoted the inhibition of XO by CSD1. AtDJ-1a only was used as a control (○). (D) Stimulation of SOD1 activity by DJ1/PARK7. SOD1 was serially diluted in the absence (□) or presence (Δ) of 3 μM DJ1/PARK7. DJ-1/PARK7 only was used as a control (○).

## Discussion

Numerous studies have demonstrated that DJ-1/PARK7 harbors antioxidant properties in relation to H<sub>2</sub>O<sub>2</sub>-induced cell death (Canet-Aviles et al., 2004; Junn et al., 2005; Kim et al., 2005; Takahashi-Niki et al., 2004). However, the molecular mechanisms underlying the anti-apoptotic properties of DJ-1/PARK7 have proven to be somewhat elusive. The aim of this study was to gain insight into the mode of action of DJ-1, taking advantage of its conserved nature and employing the model plant *A. thaliana* as a well-defined molecular tool. This has allowed us to characterize the role of DJ-1 in plants and revealed a fundamental DJ-1/PARK7 mechanism associated with cell death and stress protection.

## *Arabidopsis* harbors conserved but distinct DJ-1/PARK7 homologs

Although it has been reported that *A. thaliana* contains numerous homologs of genes encoding proteins involved in human disease (Jones et al., 2008), the finding that the *Arabidopsis* genome harbors three DJ-1/PARK7 homologs was unexpected. Amino acid comparisons of AtDJ-1a, AtDJ-1b and AtDJ-1c with human DJ-1/PARK7 showed not only the presence of amino acid residues

associated with PD conditions, but also that all three *Arabidopsis* proteins consisted of two full-length DJ-1/PARK7 polypeptides (Fig. 1A; supplementary material Fig. S1). This is striking as the DJ-1 superfamily, ranging from bacteria to mammals, harbors only one DJ-1 domain (Wei et al., 2007). The evolutionary duplication event suggested initially that plant DJ-1s might have evolved as functionally distinct proteins, as suggested for the DJ-1 superfamily of proteins, which fall into three predicted functional classes (Wei et al., 2007). However, we have shown that one of these, AtDJ-1a, shows striking molecular and cellular similarities to the human DJ-1/PARK7 protein.

The fact that *Arabidopsis* harbors three DJ-1 homologs might imply distinct functions. Alternatively, plants might have needed, because of their sessile nature and photosynthetic electron transport chain events, to evolve more than one DJ-1 protein to deal with endogenous and excessive exogenous stresses. Indeed, localization analysis demonstrates that, although AtDJ-1a localizes to the plant cytosol and nucleus (Fig. 1B,C), as shown for DJ-1/PARK7 in mammalian cells (Fig. 1E), both AtDJ-1b and AtDJ-1c localize to plastids in *Arabidopsis* (supplementary material Fig. S2). Assuming that both AtDJ-1b and AtDJ-1c have antioxidant properties, localization to plastids in plants could suggest that both proteins are involved in protection against ROS production during endogenous photosynthetic electron transport chain events (Elstner, 1991).

The finding that AtDJ-1a has identical localization patterns to human DJ-1/PARK7 implies functional similarity to mammalian DJ-1 proteins. Previous studies have demonstrated that DJ-1/PARK7 is modified in response to oxidative stress (Mitumoto and Nakagawa, 2001; Mitumoto et al., 2001) and that *DJ-1/PARK* mRNA levels increase upon trimethyltin-chloride-induced neuronal damage in mice (Nagashima et al., 2008). In agreement with these studies, we demonstrate that *AtDJ-1a* is upregulated in response to exogenous stress cues (Fig. 2), which suggests that AtDJ-1a is involved in protecting plants from excessive ROS levels in terms of a general stress response. ROS are, however, also generated by plants on purpose, acting as signaling molecules to control various processes such as growth and development, gravitropism, hormone action and retrograde signaling from chloroplasts to the nucleus (Apel and Hirt, 2004; Galvez-Valdivieso and Mullineaux, 2009). It is therefore possible that AtDJ-1a, in concert with AtDJ-1b and AtDJ-1c, might contribute to the regulatory mechanisms of a plant in fine-tuning ROS levels, thus ensuring appropriate growth and development. It has been demonstrated that ROS activates nonselective cation channels (NSCC) in plant cells; the resulting increase in Ca<sup>2+</sup> levels leads to cell elongation in roots (Foreman et al., 2003) and pollen tube growth (Malho et al., 2006). Interestingly, copper can also cause Ca<sup>2+</sup> influx into the cell cytosol (Demidchik et al., 2003), which might, in turn, stimulate AtDJ-1a; however, whether this happens is unknown. Moreover, ROS are linked to plant hormone signaling, whereby abscisic acid stimulates NADPH-oxidase-mediated ROS generation, which in turn leads to activation of Ca<sup>2+</sup>-permeable NSCCs and stomatal closure (Kwak et al., 2003; Pei et al., 2000). The prospect that AtDJ-1a might be involved in sensing and modulating ROS levels as part of normal plant development is exciting and will require further investigation.

The induction of *AtDJ-1a* in response to excess light might also suggest that *AtDJ-1b* and *AtDJ-1c* are induced by light stress to protect against elevated levels of damaging ROS in chloroplasts (supplementary material Fig. S2). In addition, it is also possible

that AtDJ-1b and AtDJ-1c are involved in both sensing and controlling ROS levels in chloroplasts, where they act as important initiators or transducers of signals from chloroplasts to the nucleus (Galvez-Valdivieso and Mullineaux, 2009).

#### Loss of AtDJ-1a leads to cell death, whereas increased AtDJ-1a levels protect plants against stress

DJ-1/PARK7 has been shown to bind nuclear Daxx, preventing Daxx translocation to the cytoplasm and activation of pro-apoptotic ASK1 (Junn et al., 2005; Waak et al., 2009). By contrast, increased cell protection has been reported for mitochondria-targeted DJ-1/PARK7 compared with wild-type or nucleus-targeted DJ-1/PARK7 (Junn et al., 2009). We characterized an AtDJ-1a loss-of-function mutant and found that, under normal unstressed conditions, AtDJ-1a-deficient plants were viable and indistinguishable from wild-type plants in terms of growth and developmental phenotypes. However, when challenged with stress-inducing conditions, such as excess light, H<sub>2</sub>O<sub>2</sub> and MV, the leaves of older AtDJ-1a-deficient plants showed the presence of large, multiple lesions, which developed as a result of localized cell death (Fig. 3). The protective role of AtDJ-1a was only evident in older tissue, with young rosette leaves exhibiting minimal consequences of DJ-1a loss of function, indicating that the response was age related. This is in good agreement with the fact that DJ-1 in humans acts as an important redox-active signaling intermediate that controls oxidative stress during age-related neurodegenerative processes (Kahle et al., 2009). Indeed, mice that lack DJ-1 are fertile and produce viable offspring, but DJ-1<sup>-/-</sup> neurons show an increased sensitivity to oxidative stress and increased cell death (Kim et al., 2005). Although ROS play a fundamental role in plants throughout development, age-related ROS-mediated effects in plants are somewhat unclear. It has, however, been shown that ROS are involved in senescence (Bhattacharjee, 2005) and that older leaves have elevated ROS levels compared with young leaves. This possibly suggests that ROS levels in senescing and aging plants have to be stringently monitored and controlled; AtDJ-1a might be involved in an age-dependent ROS-sensing mechanism.

Overexpression of AtDJ-1a was demonstrated to reverse the sensitivity of cells to stress conditions and indeed provided protection against excess light stress, and H<sub>2</sub>O<sub>2</sub> and MV treatments. Whereas wild-type plants developed few small lesions, AtDJ-1a gain-of-function transgenic plants, with elevated AtDJ-1a levels, did not develop any cell-death lesions in response to excess light treatment and only few lesions in response to H<sub>2</sub>O<sub>2</sub> and MV. These data clearly demonstrate a role for AtDJ-1a in protecting plants against oxidative stress treatments.

#### DJ-1 stimulates SOD1, but not GPX, activity in plant and mammalian cells

Our data support the conclusion that AtDJ-1a and its human counterpart protect cells from stress-induced age-related cell death. Although an indirect link between DJ-1 and SOD has been proposed, no direct molecular relationship between these two proteins has been reported. A recent report suggests that DJ-1 levels and the DJ-1 oxidation state change in response to SOD1 dysfunction in an ALS mouse model (Lev et al., 2009). It was shown that *DJ-1* mRNA and protein levels are upregulated, and that the acidic isoform of DJ-1 increased in transgenic mice with a dysfunctional *SOD1* transgene (G93A substitution) compared with the wild type (Lev et al., 2009). Furthermore, in the brains of aged DJ-1 loss-of-function mice, a possible compensatory

mechanism has been demonstrated, whereby mitochondrial manganese SOD and GPX are upregulated (Andres-Mateos et al., 2007), suggesting that SOD and GPX are involved in the mode of action of DJ-1 in the stress defense pathway.

We have shown through in vitro and in vivo studies that AtDJ-1a forms a cytosolic protein complex with CSD1 (Fig. 5A,G) and AtGPX2 (Fig. 5B). Equally, we have demonstrated that DJ-1/PARK7 interacts with both SOD1 (Fig. 5E,H) and GPX2 (Fig. 5F) in CHO cells. SOD1 and CSD1 are Cu/Zn-dependent enzymes and their activity is affected by the availability of copper. We further asked whether the activity of SOD1 and CSD1 might be affected by DJ-1 and AtDJ-1a; however, purified DJ-1 and AtDJ-1a did not enhance SOD1 and CSD1 activity. However, once the induced DJ-1 and AtDJ-1a were loaded with copper, the purified proteins had an obvious effect on the activity of SOD1 and CSD1 (Fig. 6C,D). Because the stimulation of CSD1 and SOD1 appears to be dependent on the delivery of copper from copper-loaded AtDJ-1a and DJ-1/PARK7 (Fig. 6C,D), we examined the capacity of DJ-1 and AtDJ-1a to bind copper using ITC, and confirmed that both DJ-1 and AtDJ-1a are able to bind copper (Fig. 6A,B). The data presented suggest that AtDJ-1a and DJ-1/PARK7 act as adaptor proteins that bring together SOD and GPX2 in a conformation that allows damaging superoxide to undergo SOD-mediated dismutation into oxygen and H<sub>2</sub>O<sub>2</sub>, which in turn is converted into H<sub>2</sub>O by GPX2.

#### Conclusions

Many model organisms have been employed in the study of neurodegenerative disorders, including rat, mouse, worm, fruit fly and the budding yeast *Saccharomyces cerevisiae*. *Arabidopsis* has a long history in contributing to fundamental discoveries with direct relevance to human health and disease (Jones et al., 2008), and our studies reported here suggest that plants might act as a complementary molecular tool to understand aspects of neurodegenerative disease mechanisms. We have demonstrated that the *Arabidopsis* homolog of human DJ-1/PARK7, AtDJ-1a, is involved in a general age-related stress response in plants, whereby AtDJ-1a interacts with CSD1 and AtGPX2 and enhances CSD1 activity. Similarly, our data demonstrate that DJ-1/PARK7 interacts with and stimulates SOD1 activity in mammalian cells. The finding that AtDJ-1a interacts with and stimulates CSD1 suggests that AtDJ-1a might have a dual function, acting both as an important enhancer of SOD-mediated ROS removal during stress situations and as a sensor and fine-tuner of ROS levels during normal growth and development. Understanding how AtDJ-1a and DJ-1/PARK7 split their function between these equally important roles represents an exciting future challenge.

#### Materials and Methods

##### Growth of *Arabidopsis* and mutant analysis

AtDJ-1a T-DNA insertion alleles N830498 and N551124 (Col) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC, UK). Surface-sterilized seeds were sown on half-strength MS salts (Duchefa Biochemie) media containing 0.8% phytagar (w/v) (Duchefa Biochemie), 1.0% sucrose and 10 mg/l phosphinothricin (Basta/PPT). Plants were grown on soil at 22°C under long-day conditions (16 hours light-8 hours dark). Genotyping was performed using PCR and RT-PCR with border primers LB3 (N830498) or LBb1 (N551124) and the primers listed in supplementary material Table S1. Detailed phenotypic analysis was performed throughout development. For analysis under stress conditions, wild-type *Arabidopsis* seedlings, T-DNA insertion mutants and *AtDJ-1a*-overexpressing plants were grown under long-day conditions (16 hours light-8 hours dark) for three weeks followed by treatment with excess light (280 μmol/m<sup>2</sup>/second), H<sub>2</sub>O<sub>2</sub> and MV. 10 μM of H<sub>2</sub>O<sub>2</sub> or MV, dissolved in water with 0.05% Triton X-100, was sprayed on seedlings every day for one week and phenotypes recorded.

### cDNAs and plasmid construction

All cloning was carried out using standard techniques. Primers used for cloning are listed in supplementary material Table S1. Full-length cDNAs of AtDJ-1a (At3g14990), AtDJ-1b (At1g53280), AtDJ-1c (At4g34020), CSD1 (At1g08830) and AtGPX2 (At2g31570) were isolated using RT-PCR (M-MLV reverse transcriptase, Promega) from total RNA (GenElute™ Mammalian Total RNA Miniprep Kit, Sigma). cDNAs of human DJ-1/PARK7 (IRATp970A1276D) and SOD1 (IRAU969B108D) were ordered from imaGenes (Germany).

### Expression, localization and interaction analysis

#### Expression analysis

For expression analysis, a 1358-nucleotide *AtDJ-1a* promoter region was amplified from *Arabidopsis* genomic DNA and cloned into pBADG vector containing the *GUS* gene. The plasmid was transformed into *Arabidopsis* wild-type plants using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998) and the primary transformants were selected on 15 µg/ml BASTA (Melford Laboratories). The expression pattern of AtDJ-1a was visualized using X-Gluc solution (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, Fermentas, #R0851) under various stress conditions, including excess light (280 µmol/m<sup>2</sup>/second), in the presence of 1 mM CuSO<sub>4</sub>, H<sub>2</sub>O<sub>2</sub> and MV. 10 µM of H<sub>2</sub>O<sub>2</sub> or MV, dissolved in water with 0.05% Triton X-100, was sprayed on seedlings every day prior to X-gluc staining. The growth media containing 1 mM CuSO<sub>4</sub> was accurately controlled in terms of pH.

*CSD1* expression was analysed by semi-quantitative RT-PCR (20 cycles) on seedlings of wild-type, T-DNA insertion mutants and *AtDJ-1a*-overexpressing plants without any stress treatment. *Actin8* was used as control.

#### Subcellular localization analysis

For transient localization, AtDJ-1a, AtDJ-1b and AtDJ-1c were PCR cloned into pWEN18 (Kost et al., 1998) or pWEN-NY and pWEN-CY (Maple et al., 2005). The resulting constructs were transformed into tobacco leaf cells by particle bombardment (Kost et al., 1998) and analysed for YFP fluorescence using a Nikon TE2000U inverted microscope. For stable *Arabidopsis* transformations, the AtDJ-1a-YFP cassette was subcloned into pBA002 (Kost et al., 1998). Transgenic *Arabidopsis* plants were generated using the Agrobacterium-mediated floral dip method and primary transformants were selected on 15 µg/ml BASTA (Melford Laboratories). Fluorescence in this study was analysed using a Nikon A1R confocal laser microscope.

For transient localization in mammalian cells, *DJ1/PARK7*, *SOD1* and *GPX2* cDNAs were cloned into modified versions of the vector pEGFP-C2 (Clontech) to generate N-terminal fusions to full-length enhanced GFP (pUIS-EGFP). Generated constructs were used to transfect mammalian cells.

#### Protein-protein interaction analysis

BiFC was used for protein-protein interaction analysis. AtDJ-1a, AtDJ-1b and AtDJ-1c were cloned into pWEN-NY and pWEN-CY (Maple et al., 2005). The resulting constructs were transformed and analysed following the same procedure used for transient localization analysis.

For BiFC analysis in mammalian cells, *DJ1/PARK7*, *SOD1* and *GPX2* cDNAs were cloned into pEGFP-C2 (Clontech) to generate N-terminal fusions to EGFP<sub>1-154</sub> (pUIS-NG) and EGFP<sub>155-265</sub> (pUIS-CG) (amino acid numbers in subscript). Generated constructs were transfected into mammalian cells and verification of full-length protein expression was performed by western blotting using a polyclonal anti-GFP antibody (Clontech) following standard protocols.

### Protein expression and purification

Full-length *AtDJ-1a*, *CSD1*, *SOD1* and *DJ-1/PARK7* cDNAs were cloned into pET28a followed by recombinant protein expression using standard protocols in *E. coli* Rosetta (DE3) pLysS. Purification of AtDJ-1a, CSD1, SOD1 and DJ-1/PARK7 was performed by standard affinity purification using Talon affinity resin (Clontech) as instructed by the manufacturer.

### CHO transfection

CHO-S cells were cultured in DMEM supplemented with 10% BCS, 2 mM glutamine, non-essential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, in 5% CO<sub>2</sub> atmosphere at 37°C. Cells were transfected using Lipofectamine 2000 according to the manufacturer's description (Invitrogen). Cells were fixed 24 hours post-transfection in 4% paraformaldehyde and stained with Hoechst-33342 nuclear stain prior to mounting.

### SOD1 assays

SOD1 activity was assayed using the Sigma SOD assay kit (Sigma, 19160) following the manufacturer's instructions.

### ITC

Calorimetric measurements were carried out using a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, MA). Each experiment was performed at a constant temperature of 25°C and consisted of 27 injections of 10 µl aliquots, repeated every 200 seconds. All samples were degassed by stirring under vacuum before use. Titrations of CSD1 to AtDJ1a and SOD1 to DJ-1 were performed in 10 mM Tris,

pH 7.5, 2.5 mM MgCl<sub>2</sub>. Titrations of copper (5 mM CuSO<sub>4</sub>) to DJ-1 were performed in 10 mM Tris, pH 7.5, 2.5 mM MgCl<sub>2</sub> and to AtDJ-1a in 100 mM NaAc buffer. Heat of dilution, measured by control experiments in which samples were injected into a buffer-filled cell, was subtracted. Signals recorded in each experiment were integrated using the OriginPro 7.5 software supplied with the instrument. The thermodynamic binding parameters [dissociation constant (*K<sub>d</sub>*), variations of enthalpy ( $\Delta H$ ), Gibbs free energy ( $\Delta G$ ), entropy ( $\Delta S$ ) and the number of binding sites (*n*)] were obtained by nonlinear regression of the integrated heat plots, according to the 'one set of sites' or 'two sets of sites' model of the software.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/10/1644/DC1>

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