RNAi-mediated reduction of xanthine dehydrogenase results in increased biomass of Arabidopsis seedlings

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Abstract. **Xanthine dehydrogenase (XDH), a classic enzyme involved in purine catabolism, can catalyze the formation of redox-signaling reactive oxygen and nitrogen species such as superoxide and nitric oxide. We generated transgenic plants of Arabidopsis in which XDH was knocked out by introduction of hairpin RNA-expression vector. Expression analysis by reverse transcription-PCR and in-gel staining of XDH activity revealed that transgenic lines efficiently suppressedXDH expression at the transcriptional level, demonstrating that RNA interference was successfully induced. XDH-suppressed transgenic lines exhibitedincreased biomass production during the growth of seedlings.**

Introduction

Xanthine dehydrogenase (XDH) is a ubiquitous molybdoenzyme with a central role in purine catabolism where it catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid (reviewed by Harrison, 2002). As a side reactionof xanthine oxidation, this enzyme is known to catalyze the formation of superoxide and hydrogen peroxide (Olson *et al.*, 1974). Under certain physiological conditions such as oxygen shortage, it can also reduce inorganic nitrate and nitrite to produce nitric oxide (Millar *et al.*, 1998). Owing to its catalytic activity to generate reactive oxygen species and reactive nitrogen species (RNS), recently, considerable attention has been paid to a new possible role for this classic enzyme in animal redox signaling (Harrison, 2002). In this context, however, little is understood on the physiological function of plant XDH (Sauer and Frébort, 2003).

We are interested in XDH as an RNS-generating enzyme because we have recently discovered previously unrecognized but potentially important aspects of plant nitrogen

metabolism in which RNS are almost certainly involved (Morikawa*et al.*, 2004; Sakamoto *et al.*, 2003). As an initial step to determine a possible relationship between XDH function and our findings of novel nitrogen metabolism in plants, we have generated transgenic plants (designated ko-xdh lines) of Arabidopsis in which, by RNA interference (RNAi), the expression of XDH is radically suppressed. During characterization of ko-xdh lines, we unexpectedly found that RNAi-mediated XDH reduction increased biomass of the seedlings.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype C24) was aseptically cultured in growth chambers under long-day conditions (16-h light/8-h dark) at 22°C. Surface-sterilized seeds of wild-type and transgenic plants were sown on Murashige and Skoog (MS) medium containing 0.3% (w/v) gellan gum and 1% (w/v) sucrose per pot. Two and four weeks after germination, the seedlings were recovered and weights of dry matter were determined. Statistical analysis for biomass increase was performed using Student's *t* test.

Vector construction and plant transformation

An EST clone (accession no. AV548322), that contains the complete open reading frame for an Arabidopsis XDH (AtXDH1), was obtained from Kazusa DNA Institute. Using this clone as template, we amplified the 5' part (ca. 600 bp) of the coding region of the cDNA which was subsequently cloned into the hairpin RNA-expression vector, pHellsgate 8 (Helliwell *et al.*, 2002). The resulting construct, pHG-XDH, was transferred into *Agrobacterium tumefaciens* and used to transform wild-type Arabidopsis by vacuum infiltration. To select transgenic plants (T_1 generation), the seeds from the infiltrated plants were tested for antibiotic resistance on MS solid medium supplemented with kanamycin (50 μg/ml). T-DNA insertion in kanamycin-resistant plants was verified by PCR amplification of genomic DNA.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from whole plant samples and reverse-transcribed with reverse transcriptase and oligo-d(T) primers. A pair of gene-specific primers was used to amplify cDNA fragments for each *AtXDH* transcript. The expected sizes of the amplified fragments are 456 bp and 563 bp for *AtXDH1* and *AtXDH2*, respectively. The subunit A of V-type ATPase (*AtVHA-A*) served as an internal control.PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE; 8% gel).

In-gel staining of XDH activity

Soluble protein was extracted from whole plant samples and an aliquot (30 μg) was separated by PAGE (7.5% gel) under non-denaturing condition. Following PAGE, XDH activity was detected *in situ* using hypoxanthine as substrate and nitro blue tetrazolium as chromogenic reagent for positive staining.

Results and Discussion

Generation of ko-xdh plants

Because Arabidopsis genome possesses two copies of XDH gene (*AtXDH1* and *AtXDH2*), we attempted to inactivate both genes simultaneously by RNAi rather than to obtain T-DNA insertional mutant lines. To this end, we constructed the hairpin RNA-expression vector withthe partial *AtXDH1* cDNA segment that is highly homologous to the corresponding region in *AtXDH2*. Seeds from infiltrated plants were allowed to germinate and grow on selective medium, and kanamycin-resistant plants (T_1 generation) were examined by genomic PCR for the integration of T-DNA. Sixteen individuals were confirmed to carry T-DNA, and from which five transformants (ko-xdh1 to ko-xdh5) were selected, self-fertilized and used for further experiments.

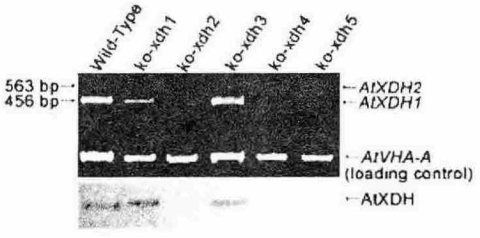

Figure 1.
RT-PCR analysis of *AtXDH1* and *AtXDH2* expression (upper image) and XDH activity (lower image) in seedlings of wild-type and transgenic plants of *Arabidopsis*. *AtVHA-A* was used as a loading control in RT-PCR.

Expression of AtXDH1 *and* AtXDH2 *in ko-xdh plants*

Fig. 1 shows the expression of *AtXDH1* and *AtXDH2* in the five independent lines of ko-xdh plants (T_2 generation) that was analyzed by RT-PCR and in-gel staining for XDH activity. In comparison to wild-type plants, *AtXDH1* mRNA levels were drastically reduced in ko-xdh2, ko-xdh4, and ko-xdh5, whereas the mRNA levels in ko-xdh1 and ko-xdh3 were not. *AtXDH2* mRNA levels were extremely low in all the tested line including wild-type plants, and therefore, it is not clear whether the expression of *AtXDH2* was indeed suppressed by transformation(Fig. 1, upper image). Consistent with the results of RT-PCR, XDH activity was not detected in ko-xdh2, ko-xdh4 and ko-xdh5 (Fig. 1, lower image), demonstrating that introduction of hairpin RNA-expression vector efficiently reduced XDH expression. These results also indicate that, in Arabidopsis, *AtXDH1* is the dominant form

but its expression is not probably essential under normal growth condition. In-gel activity staining with T3generation showed persistent absence of XDH activity in ko-xdh2, ko-xdh4 and ko-xdh5 lines, thus RNAi-mediated suppression of XDH is an inheritable trait (data not shown).

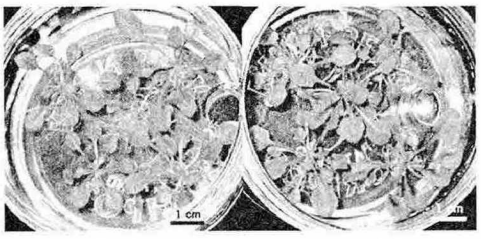

Figure 2.
Growth of 4-week-old seedlings of wild-type and ko-xdh5 plants on MS solid medium. Five seeds per pot were germinated and grown in growth chambers under long-day conditions (16-h light/8-h dark) at 22°C.

Growth performance of ko-xdh plants

XDH-suppressed ko-xdh plants exhibited no distinguishable differences from wild-type plants with respect to gross morphology under normal growth condition. One noticeable difference, however, is that these transgenic lines increased biomass during the growth of seedlings (Fig. 2). When compared to wild-type plants, two and four weeks after germination, homozygous T3 generation of ko-xdh5plants (with a single T-DNA insertion) increased biomass production by 20% and 32%, respectively, on the dry weight basis (significant at $P < 0.001$ for 2-week-old plants ($n = 14$) and at $P < 0.05$ for 4-week-old plants ($n = 24$)). Seeds from both types of plants germinated equally well following imbibition. Therefore, the difference in biomass production between wild-type and transgenic plants could be attributable to the difference in growth performance. These results might suggest that XDH could be a target for molecular breeding to improve growth performance, especially of young plants. Currently, we are investigating the molecular mechanism responsible for the RNAi-mediated enhancement of the growth of transgenic seedlings in relation to XDH function.

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