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BRIEF COMMUNICATION

Photosynthetic gene expression in black willow under various soil moisture regimes

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Abstract

This study was the first attempt to extract RNA from black willow (*Salix nigra* Marshall) that contains numerous secondary products and to examine the photosynthetic gene expression of black willow under a wide range of soil moisture regimes. Black willow cuttings were grown under control, continuous flooding, periodic flooding and periodic drought for 42 d. A modified lithium chloride precipitation method was used for RNA extraction. Results of real-time polymerase chain reaction showed reduced gene expression of oxygen evolving complex, large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenasse and ferredoxin on day 7 as well as the latter two on day 14 in response to flooding. Therefore, decreased expression of these three genes may have contributed to the observed reduced photosynthetic capacity in response to flooding.

Additional key words: ferredoxin, large subunit of Rubisco, oxygen evolving complex, *Salix nigra*.

Black willow (*Salix nigra* Marshall) naturally occurs in floodplains and riparian zones of the southeastern United States (Mitsch and Gosselink 1993), where it is subjected to dynamic hydrologic conditions. Depending on the slope and depth to base flow, black willow may be exposed to continuous flooding, periodic flooding or periodic drought. It is known that photosynthesis and growth of black willow under both continuous flooding and periodic drought are limited by stomatal closure and some non-stomatal factors such as decreased leaf chlorophyll content and photosystem activity measured as increased fluorescence yield in dark adapted plants (Pezeshki *et al.* 1998, Li *et al.* 2004). However, no data are available on how photosynthetic gene expression responds to these conditions and whether photosynthetic enzymes are also among the contributing factors to photosynthetic decline. Such data may help understand the molecular mechanisms involved in plant responses to flooding and/or drought. Such information may also provide guidance for molecular strategies, such as development of transgenic plants, which have been used in an attempt to increase waterlogging and/or water stress tolerance in plants (Dennis *et al.* 2000, Cherian *et al.* 2006).

Under environmental stresses, changes in gene expression take place, with both up- and down-regulation occurring. Cellular and molecular biological studies have focused on identifying genes being up-regulated by environmental stresses in limited model species of flowering plants, such as *Arabidopsis thaliana* and some crop plants (Sachs *et al.* 1980, Bray 2002). However, very limited data are available on the expression of genes

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Abbreviations: C - control; CF - continuous flooding; Fd - ferredoxin; OEC - oxygen evolving complex; PCL - phenol:choroform:isoamyl alcohol; PD - periodic drought; PF - periodic flooding; PPFD - photosynthetic photon flux density; rbcL - large subunit of Rubisco; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenasse.
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that are down-regulated by environmental stressors (Williams *et al*. 1994, Cheng *et al*. 1998, Vu *et al*. 1999, Henmi *et al*. 2004). For instance, Liu *et al.* (2006) reported reduced steady-state transcript levels of chloroplast gene *psbA, psbD* and nuclear gene *cab* during progressive water stress.

The objective of this study was to examine the photosynthetic response of black willow under flooding and drought conditions at the molecular level. Three photosynthetic genes studied were coding for these proteins: oxygen evolving complex (OEC), ferredoxin (Fd) and large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase, Rubisco (rbcL). We expected that decreased expression of these three genes could be one of the reasons for the photosynthetic decline observed after continuous flooding or periodic drought as reported in a previous study (Li *et al*. 2004). No data have been reported on expression of genes involved in photosynthesis in wetland plants under well-defined soil flooding or drought conditions. In addition, this study was the first to attempt to extract RNA from black willow. This species, like many other woody species, presents a new set of challenges for molecular biology due to the numerous secondary products present in their tissues (Palo 1984). Therefore, the present study also aimed to develop a method that would work reliably for RNA isolation from woody plants and would pave the way for future studies in the field.

Black willow (*Salix nigra* Marshall) cuttings were collected from a localized population on the Loosahatchie River in western Tennessee, USA, in January of 2003 during the dormancy of the plants. Each cutting was 1.0 - 1.2 cm in diameter at the base and 33 cm in length. Cuttings were planted in the laboratory immediately after collection. Thirty cuttings were planted into pots (one cutting per pot, 20 cm deep, 20 cm wide). Pots were filled with the mixture of sand and soil (2:1 v:v). Environ-mental conditions (a day/night temperature of 25/20 °C, a 16-h photoperiod, photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1} at the top of plants, well watered and well drained soil moisture condition) were maintained during whole growth of plants. Treatments were initiated on February 23 when plant cuttings were 35-d-old.

Sixteen plants selected for their uniformity were used to test the responses of willow cuttings to four soil moisture regimes: control (C), continuous flooding (CF), periodic flooding (PF), and periodic drought (PD) (as described in Li *et al*. 2004). The experiment followed a completely randomized design. Each treatment consisted of a total of four replicate cuttings. Plant cuttings were grown under each treatment for 42 d.

Leaf samples were collected on day 0, 7, 14, 21, 28, 35 and 42 (four samples per treatment per day) and stored at -80 ºC until further processed. On day 7 and 14, no samples were collected for PD since no sign of water deficit in the soil was shown (*i.e.*, soil water potential not in the range of -0.5 to -0.8 MPa). Leaf tissue (~ 1 g) was ground to a fine powder in liquid nitrogen and immediately added to 10 cm^3 lysis buffer (100 mM Tri-HCL pH 9.0, 50 mM EDTA, 200 mM NaCl, 2 % sodiumdodecyl sulphate (SDS), and 50 mM 2-mercapto-ethanol) and 10 cm^3 PCL (phenol:choroform:isoamyl alcohol 25:24:1). The sample extract was centrifuged (14 000 *g*) and re-extracted with an equal volume of PCL twice and centrifuged again (14 000 *g*). 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added to the top aqueous phase and the solution was centrifuged again. The pellet was dissolved in 1/2 the original lysis buffer volume RNase free water and centrifuged (14 000 *g*) with an equal volume of cold 4 M LiCl. Before the pellet dried, 1/10 to 1/4 the original volume of water was added to dissolve the pellet. An equal volume of cold 4 M LiCl was added and then the solution was centrifuged again (14 000 *g*). Supernatant was decanted and drained as before. RNA was dissolved in RNase free water. Sodium acetate was adjusted to 0.3 M and 2.5 volume of ethanol. The RNA was stored at -80 ºC.

The following components were added to a nuclease-free microcentrifuge tube: 0.001 cm^3 50 µM oligo $(dT)_{12-18}$, 0.001 cm^3 10 mM dNTP and 1.5 µg RNA. Adequate amount of distilled water was then added to make the total volume of the solution 0.012 cm^3. The mixture was heated for 5 min at 65 ºC and quickly chilled on ice. After chilling, 0.004 cm^3 5× First-Strand Buffer, 0.002 cm^3 0.1 M dithiotreitol (DTT), 0.001 cm^3 RNaseOUT and 0.001 cm^3 M-MLV RT were added. The mixture was incubated at 37 °C for 52 min and then inactivated by heating at 70 ºC for 15 min. The cDNA was ready to be used as a template for amplification in real-time PCR.

Gene specific primers to test expression levels by quantitative PCR were as follows: rbcL forward primer (5'GGGATTCACCGCAAACACTACCTT3') and reverse primer (5'TACCGCGGCTTCGATCTTTTTCA3'); OEC forward primer (5'GATCGCCACCGCTCCTTCTC3') and reverse primer (5'GTTAGCCGTTCCAGTTCCTTTCAC3'); Fd forward primer (5'GCCCCTTCGCCTCGCCTCTC3') and reverse primer (5'GTCGCCGGTGATCCTGGTGTTGA3'); The housekeeping gene was tubulin: forward primer (5'ATTCCCCCTGCTTATTCAAAAC3') and reverse primer (5'ACCCGCAGTAGCATTCAAGTAA3').

Following components were mixed: 0.025 cm^3 iQ SYBR Green Supermix (*Bio-Rad Laboratories*, Hercules, CA, USA), 0.001 cm^3 of forward primer, 0.001 cm^3 of reverse primer, 0.018 cm^3 sterile water and 0.005 cm^3 50 µg cm^{-3} cDNA template. Real-time PCR was then performed using the *Bio-Rad iCycler*. Relative gene expression was quantified by the comparative Ct method.

One-way *ANOVA* followed by Tukey procedure (*SPSS 11.5*) was used to test differences in means of fold change for rbcL, Fd and OEC between treatments on each sampling day and *t*-test was used if results of gene expression were available for only two treatments. Differences were considered significant at $P < 0.05$.

Table 1. Fold change for *rbcL*, *Fd* and *OEC* for black willows under control (C), continuous flooding (CF) and periodic flooding (PF) on day 7 and 14. Each value is the mean for four replications ± SE. Significant differences are shown across treatments on each day using different letters.

Treatment	Day 7			Day 14		
	rbcL	*Fd*	*OEC*	*rbcL*	*Fd*	*OEC*
C	1.62 ± 0.42 a	1.07 ± 0.18 a	3.73 ± 0.48 a	3.25 ± 0.47 a	0.23 ± 0.07 a	1.00 ± 0.42 b
CF	0.33 ± 0.09 b	0.35 ± 0.08 b	2.43 ± 0.33 b	0.23 ± 0.29 b	0.09 ± 0.05 b	2.03 ± 0.57 a
PF	0.29 ± 0.12 b			0.44 ± 0.21 b		

RNA concentration ranged from 0.153 to 3.561 g dm^{-3} for all samples across treatments. The purity of total RNA fractions was judged by the ratios of A_{260}/A_{280}. Our data showed that A_{260}/A_{280} for most samples, except those on d 0 and 7, was lower than 1.50. This suggested that secondary compounds contaminated those RNA fractions. However, the results of the integrity of the RNA examined by electrophoresis using *Agilent* bioanalyzer indicated that intact RNA bands were detected in most samples.

Real-time PCR indicated that *rbcL* gene expression was different between C, CF and PF plants on day 7 (P = 0.013). It was down-regulated in the latter two treatments. *Fd* and *OEC* transcript levels also were reduced in CF cuttings as compared with C cuttings (P = 0.034 and 0.042, respectively, Table 1). On day 14, *rbcL* and *Fd* expression exhibited a similar trend to that seen on day 7. However, increased *OEC* transcript levels were observed in CF cuttings on day 14 as compared with those under C (P = 0.029) (Table 1). Results of the comparison of gene expression among other samples were inconclusive.

Our study demonstrated that *rbcL* gene expression in black willow was down-regulated by both CF and PF treatments on day 7. Transcript levels of *Fd* and *OEC* also showed reduction in CF cuttings as compared with C. On day 14, *rbcL* and *Fd* showed the same trend, which indicated that the prolongation of the flooding stress continued to negatively affect the expression of these two genes. Since Rubisco is critical enzyme of the Calvin cycle, flood-induced reductions of net photosynthesis in black willow (Li *et al*. 2004) may be at least partially due to decrease in *rbcL* gene expression of as shown in this study. In addition, OEC and Fd are important enzymes in the electron transport chain. Therefore, decreased gene expression of *Fd* on day 7 and 14 as well as *OEC* on day 7 could also be responsible for the reduction in photosynthesis in black willow. Previous reports (Li *et al.* 2004) and present results suggest that flooding influences plant performance at many levels including the molecular one, which could contribute to reduced photosynthetic capacity in response to low soil oxygen conditions.

An increased *OEC* gene expression under CF treatment was observed on day 14, suggesting the resilience of *OEC* to low soil oxygen conditions. However, no recovery in net photosynthesis was observed in flooded black willow at the same time (Li *et al*. 2004). An increased *OEC* gene expression on day 14 in the present study was therefore either insufficient to improve the photosynthetic capacity or was offset by other limiting factors, *e.g.* stomatal closure (Li *et al*. 2004) and decreased *rbcL* and *Fd* gene expression. Increased *OEC* gene expression under flooding has not been reported in the literature, thus merits further investigation.

The analysis of the changes in gene expression of *OEC* and *Fd* under PF on day 7 and 14 together with *rbcL*, *OEC* and *Fd* under C, CF, PF and PD on day 0, 21, 28, 35 and 42 was not possible due to the problems we experienced with purity and integrity of the extracted RNA. Therefore, a better protocol needs to be developed for high-quality RNA from black willow leaves. If the modified LiCl method still proves to be the best for this species, less than 1 g of leaf tissue might work better. In addition, more caution should be taken to avoid the degradation of RNA due to release of RNases. Repeated clean-up might be also necessary to minimize the contamination of secondary compounds. It is known that willows contain phenolglycosides as main secondary compounds (Palo 1984). On the other hand, the problems we experienced during real-time PCR procedure had to do with designing a specific tubulin primer for black willow, as there are very little molecular sequences available for willows.

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Wojtkowski, P.A.: **Introduction to Agroecology: Principles and Practices.** - Haworth Food & Agricultural Products Press, An Imprint of The Haworth Press, New York - London - Oxford 2006. 404 pp. USD 79.95. ISBN 13: 987-1-56022-317-7.

Agroecology is a compilation of views on the way how to exploit harmoniously land and nature for production of food, fuel, fiber, and other resources essential for the life of people. Contemporary agroecology mainly explores interrelationships between the nature and agricultural activities of the people. The book "Introduction to Agroecology: Principles and Practices" of Prof. P.A. Wojtkowski provides to the readers a wide spectrum of information in readable and well-arranged form.

According to the author, agroecology should be more than the raising of crops. Through the various associations, agroecology should add new insights, promotes an interchange of ideas how not to exploit the nature and enhances the agriculture in different regions of the world.

The book is divided into 18 chapters. The main goals of the agroecology are specified in the first chapter, and mainstreams and alternative forms of agriculture are characterized. Further, the attention is given to the questions, concerning the territorial basis and the landscape planning, biodiversity, agrobiodynamic conceptions, problems of the certainty-sustainability, climatic risks and productive threats – drought, cold, heat, and soil depletion. Following chapters solve the problems of the agroecosystems, inputs to the agriculture, use of agrochemicals, weeds, pests and diseases protection. Furthermore, the importance of trees in agriculture is emphasized. Within the frame of each chapter not only technological, but also ecological and economical aspects are solved. One chapter is also given to the social and community questions, concerning the quality of life, challenges and economic gains of agroecology. In the last chapters, problems of the ecosystems integration, intensity exploitation of the soil and agricultural landscape are solved.

Advantage of the book lies in its complexity and wide range, comprising of not only questions and problems of the long- and short-term risks related to the farming on the land in the agricultural landscape, but also social and economical aspects. Book aids to the reader to orientate oneself in the full range of the interrelationships and interface among the nature and agriculture, shows the agroecology in its entity and advises to the reader, how to do it and how to achieve "friendly" and harmonious exploitation of the nature and agricultural landscape.

The book is interesting by its conception and complexity and makes a useful source of information for all readers from university teachers and researchers to farmers and other sustainable producers.

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