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Grafting improves drought tolerance by regulating antioxidant enzyme activities and stress-responsive gene expression in tobacco

Jianjun Liu [a,b], Junqi Li [a], Xinhong Su [c], Zongliang Xia [a,*]

[a] Henan Agricultural University, Zhengzhou 450002, PR China
[b] Zhengzhou Branch, Henan Tobacco Company, Zhengzhou 450001, PR China
[c] Henan Tobacco Company, Zhengzhou 450008, PR China

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ABSTRACT

Grafting has been used to induce abiotic stress tolerance in horticultural crops recently. However, few studies have come out with the effects of grafting and its possible molecular mechanism under drought conditions in tobacco. In this study, using the drought-tolerant cultivar Nongda202 as a rootstock and the drought-sensitive cultivar K326 as a scion, we exposed non-grafted (K326/K326), self-grafted (K326/K326) and rootstock-grafted (K326/Nongda202) tobacco plants to drought stress over a 12-day period, and investigated their differential physiological and molecular responses to drought stress. Our results showed that drought-induced inhibition of growth was significantly alleviated in rootstock-grafted plants, as was evidenced by the physiological indexes, such as much higher SOD and CAT activities, higher levels of proline accumulation and lower levels of lipid peroxidation. Further expression analysis by real-time PCR indicated that rootstock-grafting affected transcripts of stress-responsive genes *NtAREB*, *NtCDPK2*, *NtLEA5* and *NtERD10C* in a prompt and lasting manner. Together, these results demonstrated that grafting with rootstock genotypes tolerant to drought could improve drought stress tolerance in tobacco by regulating antioxidant enzyme activities and stress-responsive gene expression. This study will facilitate to broaden our understanding of the regulatory machinery of grafting, and will accelerate potential application of the grafting technique in improving biotic and abiotic stress tolerance in crop plants.

1. Introduction

Drought is one of the most common environmental stress factors that influence plant growth and development, and has become the primary cause for reductions in crop yields (Boyer, 1982; Luo, 2010). Drought stress can lead to a series of physiological and biochemical responses such as stomatal closure, cell division suppression, photosynthesis inhibition, and reactive oxygen species (ROS) accumulations (Shinozaki and Yamaguchi-Shinozaki, 2007). When subjected to drought stress, plants can respond and adapt to the adverse conditions by triggering antioxidant defense system to maintain cellular function (Hasegawa et al., 2000; Kawasaki et al., 2001; Zhu, 2002).

Grafting began in the late 1920s, and was initially applied to counteract soil pathogens such as *Fusarium oxysorum* (Ke and Saltveit, 1988). Recently, the technique has been widely used to improve abiotic stress tolerance and nutrient uptake, going beyond resistance to soil-borne pests and diseases. For example, grafting with tolerant rootstocks protects plants against heat stress in tomato and increases resistance to chilling and oxidative stresses in cucumber (Rivero et al., 2003; Zhou et al., 2007; Li et al., 2014). Grafting also enhances tolerance to salt stress in tomato, melon and cucumber (Romero et al., 1997; Santa-Cruz et al., 2002; Fernandez-Garcıa et al., 2002; Estan et al., 2005; Ruiz et al., 2006; Martinez-Rodriguez et al., 2008). Interestingly, recent studies have shown that grafting can induce resistance against heavy metal toxicity, enhance nutrient uptake, and increase synthesis of endogenous hormones (Ahmedi et al., 2007; Dong et al., 2008; Rouphael et al., 2008; Huang et al., 2013). In relation to drought tolerance, the responses of grafted plants to drought have been studied in orange, apple and tomato (Erismann et al., 2008; Bauerle et al., 2011; Sánchez-Rodríguez et al., 2012). However, conclusions from these horticultural plants may not be applicable to other species, so research on species-specific responses to drought is needed.

Tobacco is an important crop as well as a model plant system, and its productivity is vulnerable to drought. To date, no reports have

Abbreviations: ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; ABA, responsive element-binding protein; CDPK2, calcium-dependent protein kinase 2; LEA5, late embryogenesis abundant 5; ERD10C, early responsive to dehydration 10.

* Corresponding author. Tel.: +86 371 63579676; fax: +86 371 63555790.

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come out with the effects of grafting on drought in tobacco. Here, we hypothesized that grafting with rootstock genotypes tolerant to drought may induce drought tolerance in tobacco by triggering antioxidant system and stress-responsive gene expression. To test this idea, using a drought-tolerant cultivar Nongda202 as a rootstock and a drought-sensitive cultivar K326 as a scion, we exposed non-grafted, self-grafted and rootstock-grafted tobacco plants to drought for about two weeks and investigated the effects of grafting and its possible mechanism at physiological and molecular levels under drought conditions.

2. Materials and methods

2.1. Plant materials and drought treatment

Nicotiana tabacum cv. K326 (drought-sensitive) was used as a scion and Nongda202 (drought-tolerant) was selected as a rootstock in this study. Tobacco seeds were surface disinfected in 75% ethanol for 3 min and in 5% sodium hypochlorite for 15 min. The germinated seeds were transferred to soil, and seedlings were grown at approximately 28 °C with 60% relative humidity, a photoperiod of 16 h/8 h (day/night) and light intensity of 300 $\mu mol\, m^{-2}\, s^{-1}$ in the greenhouse. Grafting was performed when seedlings have developed 7–8 true leaves (about five weeks old) using the procedure described previously (Santa-Cruz et al., 2002). Non-grafted (K326) and self-grafted (K326/K326) plants were included as controls. In order to maintain a high humidity level and facilitate graft formation, seedlings were covered with a transparent plastic lid and were left in the shade for 24 h. The plastic was opened slightly every day to allow reduction in relative humidity and it was removed 3 days after grafting. The grafted plants, together with controls, were grown under normal conditions in the greenhouse for another three weeks for drought treatments.

Drought stress was given to eight-week-old potted plants by withholding water to reach the soil moisture about 30%, and maintained by watering properly during 12 days. This treatment, in pilot experiments, had been shown to constitute significant stress and could not lead to severe growth inhibition at this developmental stage. The control pots were irrigated daily. Soil moisture was measured daily using a Soil Moisture Meter (ECA-SW1, TuoPu Bio Co., Qingdao, China). The experimental design was a randomized complete block with 6 treatments (K326 un-grafted, K326 self-grafted, K326/Nongda202 well-watered 100% and water stress 30%) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each. Leaf samples from control or treated plants were collected and frozen immediately in liquid N_2, and kept at −80 °C until used.

2.2. Antioxidant enzymes extraction and activity assay

Frozen leaves (0.5 g) were crushed into powder in liquid N_2. Crude proteins were extracted by homogenizing the powder in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C and the resulting supernatants were used for the determination of enzymatic activity. Protein concentration was determined using a coomassie brilliant blue with bovine serum albumin as the standard (Bradford, 1976). Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the methods described by Giannopolitis and Ries (1977).

One unit of SOD activity was defined as the amount of enzyme inhibiting NBT reduction by 50%.

CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240 nm for 3 min (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 200 ml of enzyme extract in a 3 ml volume. All spectrophotometric analyses were conducted as described previously (Xia et al., 2012a). One unit of CAT was defined as the amount of enzyme catalyzing the decomposition of 1 μmol of H_2O_2 per minute.

2.3. Determination of proline, MDA and chlorophyll contents

Proline content was determined according to the method of Bates et al. (1973). 100 mg of frozen plant material was homogenized in 1.5 ml of 3% sulphosalicylic acid and centrifuged. 100 μl of the extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) for 1 h at 100 °C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 1 ml toluene and was measured at 520 nm. The proline content was calculated according to a standard curve.

MDA content was determined as described previously (Draper and Hadley, 1990). Leaves were homogenized in 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 3000 × *g* for 10 min. Supernatants were collected and reacted with an equal volume of 0.67% (w/v) thiobarbituric acid (TBA) in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 3000 × *g* for 10 min. The absorbance of supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm and 450 nm.

Total chlorophyll content was determined according to the method of Arnon (1949).

2.4. Real-time PCR analysis

To understand molecular regulation of rootstock-grafting during drought stress, four representative stress-responsive genes (*AREB*, *CDPK2*, *LEA5*, and *ERD10C*) were selected and their transcript levels were monitored using real-time PCR analysis. The regulatory genes *AREB* and *CDPK2*, and the functional genes *LEA5* and *ERD10C* have been shown to respond to drought stress in tobacco (Xia et al., 2013); moreover, *AREB* and *CDPK* are involved in stress responses during heterogarfting in grapevine (Cookson et al., 2014). Real-time PCR was performed with the RNA samples isolated from eight-week-old grafting plants harvested during the 12 days of drought stress. Total RNA isolation and reverse transcription were performed as described previously by us (Xia et al., 2012b). PCR amplification was performed with primers specific for various stress-responsive genes: for *LEA5* (accession no. AF053076), the primers were 5′-TTGTTAGCAGGCGTGGGTAT-3′ and 5′-CTCTCGCTCTTGTTGGGTTC-3′; for *ERD10* (accession no. AB049337), the primers were 5′-ACGGACGAATACGGCAATC-3′ and 5′-TCTCCTTAATCTTCTCCTTCATCC-3′; for *CDPK2* (accession no. AJ344156), the primers were 5′-AGGTGAGCTTTTCGATAGGATTATT-3′ and 5′-ACTTCTGGTGCAACATAGTAAGGAC-3′; for *AREB*, (accession no. BAB61098), the primers were 5′-TCTTCACAGCAAAAGCCTCA-3′ and 5′-GTGACCCCATTATGCAATCC-3′. Criteria for designing primers were a primer size between 22 and 25, an optimal T_m at 60 °C, and a product size ranging from 200 bp to 250 bp. Data were obtained using the IQ5 optical system software (Bio-Rad). Data were normalized to the reference gene *Actin* (accession no. AF15640) transcripts using primers Act-F: 5′-TGGCATCACACTTTCTACAA-3′ and Act-R: 5′-CAACGGAATCTCTCAGCTCC-3′, which produces a 228 bp product. For the entire qRT-PCR assay, three technical replicates were performed for each experiment and the expression of each gene was investigated in three biological replicates.