Journal of Plant Physiology 198 (2016) 39–48

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Biochemistry

SWAPDT: A method for Short-time Withering Assessment of Probability for Drought Tolerance in *Camellia sinensis* validated by targeted metabolomics

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ARTICLE INFO

Article history:
Received 19 November 2015
Received in revised form 6 April 2016
Accepted 11 April 2016
Available online 19 April 2016

Keywords:
Abiotic stress
Amino acids
Camellia sinensis
Carbohydrates
Drought tolerance
GC–MS
LC–MS
Metabolite profiling
Short-time withering
Targeted metabolomics

ABSTRACT

Climate change is causing droughts affecting crop production on a global scale. Classical breeding and selection strategies for drought-tolerant cultivars will help prevent crop losses. Plant breeders, for all crops, need a simple and reliable method to identify drought-tolerant cultivars, but such a method is missing. Plant metabolism is often disrupted by abiotic stress conditions. To survive drought, plants reconfigure their metabolic pathways. Studies have documented the importance of metabolic regulation, i.e. osmolyte accumulation such as polyols and sugars (mannitol, sorbitol); amino acids (proline) during drought. This study identified and quantified metabolites in drought tolerant and drought susceptible *Camellia sinensis* cultivars under wet and drought stress conditions. For analyses, GC–MS and LC–MS were employed for metabolomics analysis.%RWC results show how the two drought tolerant and two drought susceptible cultivars differed significantly ($p \leq 0.05$) from one another; the drought susceptible exhibited rapid water loss compared to the drought tolerant. There was a significant variation ($p < 0.05$) in metabolite content (amino acid, sugars) between drought tolerant and drought susceptible tea cultivars after short-time withering conditions. These metabolite changes were similar to those seen in other plant species under drought conditions, thus validating this method. The Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) method presented here provides an easy method to identify drought tolerant tea cultivars that will mitigate the effects of drought due to climate change on crop losses.

1. Introduction

1.1. Camellia sinensis

Tea made from the leaves of *Camellia sinensis*, as green or black tea, has been drunk as a mild stimulant due to the caffeine content, since time immemorial! Tea consumption has been increasing in recent years, due to the health promoting effects associated with its rich polyphenol content (Tong et al., 2014). Plant response to stress is manifested by physiological and metabolomic responses (Maritim et al., 2015). This enables the screening and selection of tea cultivars resistant to drought stress, through organic osmolytes accumulation. Most osmolytes are secondary metabolites, and tea contains high polyphenol amounts (Cheruiyot et al., 2007). No metabolites have been investigated in *C. sinensis* in relation to drought. However, several metabolites have been documented in literature relative to drought stress in other plant species. This study focuses on polyphenols, flavonoids, amino acids and sugars.

1.2. Plant metabolomics

When plants are subjected to abiotic stress conditions, metabolic enzyme inhibition or substrate shortage, etc. disrupts plant metabolism resulting in metabolic pathway reconfiguration, ensuring plant survival (Hamanishi et al., 2015). Plants have established innumerable strategies in response to drought (Ogbaga et al., 2014). A common leaf response to drought stress involves both physical and morphological changes. Studies on leaves in connec-

Abbreviations: DS, drought susceptible; DT, drought tolerant; GC, gas chromatography; LC, liquid chromatography; PCA, principal component analysis; P5CS, pyrroline-5-caboxylate synthetase; GR, glutathione reductase; GSA, glutamate-semialdehyde; RWC, relative water content; TPC, total polyphenol content.

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http://dx.doi.org/10.1016/j.jplph.2016.04.004
0176-1617/

tion with drought tolerance have been documented in different plants but not in *C. sinensis*. Several studies have been conducted on plants subjected to drought stress, showing the importance of metabolic regulation, i.e. accumulation of osmolytes in a response to drought stress (Slama et al., 2015). Hyperosmotic stress produces osmolytes which include polyols and sugars (mannitol, sorbitol and trehalose) and amino acids (proline and betaine) (Weckwerth et al., 2004). These compounds are water soluble and non-toxic at high concentrations. They stabilise protein structure while decreasing protein-solvent interactions during water deficit, repair damaged tissues and support growth (Ruan and Teixeira da Silva, 2011). Metabolomic changes in *Arabidopsis* leaves under drought conditions show that proline, raffinose, γ-amino butyrate (GABA) and Krebs cycle intermediates accumulate in response to drought stress (Urano et al., 2009). Proline accumulation is integral for a cell's adaptation to hyperosmotic stress. It decreases water potential resulting in osmotic adjustment and maintaining of cell turgor. A large number of plant species accumulate proline in response to osmotic stress. Proline biosynthesis is activated under dehydration conditions with pyrroline-5-caboxylate synthetase (P5CS) as the target enzyme. Alternative pathways responsible for proline upregulation under drought stress include the pentose phosphate pathway. Proline biosynthesis also regulates cytosolic pH and $NADP^+$ synthesis, which are key in stimulating the pentose phosphate pathway (Hare and Cress, 1997).

Glucose and fructose levels increase in apple trees subjected to drought conditions while starch levels decrease (Ayaz et al., 2000). This suggests that both sugar alcohols and monosaccharaides play a key role in osmotic adjustment (Pandey et al., 2004). The decrease in starch concentration can be attributed to the fact that drought stress reduces the rate of photosynthesis. Carbohydrate metabolism is linked to photosynthesis, making it pivotal in the stress tolerance. Monosaccharaides such as glucose and fructose represent 38% (w/w) and sucrose 62% (w/w) of the total soluble sugars (daily average) found in watered plants, and 53% (w/w) and 47% (w/w) respectively in drought subjected plants (Rodrigues et al., 1993). As drought exposure prolongs, a reduction in the abundance of the two sugars occurs because they are increasingly being converted into protective sugars (Farrant et al., 2009).

1.3. Current drought tolerance assessment

Recurring droughts and future climate change necessitate the selection of DT tea cultivars for a sustainable tea industry. The current method for drought tolerance assessment in *C. sinensis* is visual assessment of leaf wilting and scoring on a five-point scale. This is done under field conditions, during natural droughts that occur every three to seven years. This method is subjective and poorly reproducible due to environmental conditions. An accurate and reproducible method is required to help tea breeders classify new cultivars as DT or DS. The new method should be independent of natural droughts and subjective evaluations. This inspired us to develop a short-time withering method and objective measurement of RWC, as a surrogate marker for calculating the probability of drought tolerance of new tea cultivars. This method is based on leaf RWC by mass balance as described below. The modulation of leaf metabolites (amino acids, sugars and flavonoids) between wet and drought conditions, have been determined in various plant species (as described above), but never in tea. Thus, modulation of tea leaf metabolites will be measured to validate the new method. We anticipate that the metabolite changes found in other plant species, under prolonged drought conditions, will occur in plucked tea shoots during the new short-time withering method. The main focus throughout this study was to identify, quantify and validate the metabolites in DT and DS tea (*C. sinensis*) cultivars affected by drought stress using the Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) method.

2. Materials and methods

Five biological replicates from each of the two drought tolerant cultivars (PC168 and PC153) and five biological repeats each from the two drought susceptible cultivars (PC105 and PC165) developed at the Tea Research Foundation for Central Africa in Malawi grown in pots under shade net at the University of Pretoria experimental farm in Hatfield, Pretoria and four DT cultivars (SFS 150, TN 14-3, 301/4 and 303/577) and four DS cultivars (AHP S15/10, TRFK 371/8, SC12/28 and Ejulu) grown at the Tea Research Institute in Kenya were used in this study.

2.1. Polyphenol extraction and content determination

Before extractions, fresh leaves from each cultivar growing under a shade net were microwave dried for five min, which in the process deactivated the oxidizing enzyme polyphenol oxidase. A coffee grinder was used to grind the dried leaves and sieved through a 355 μm stainless-steel sieve and stored at 4 °C in plastic zip-lock bags prior to extracting polyphenols. International Organization for Standardization (ISO) extraction method was used as is described in the ISO document 14502-1: 2005. Briefly, 0.200 ± 0.001 g of each sample was weighed out on a Mettler Toledo analytical balance (Microcep, South Africa) and transferred into a glass extraction tube. A five ml volume of 70:30 methanol (Merck, South Africa): water (v/v) at 70 °C was added to each extraction tube, stoppered and vortex mixed for approximately five seconds before placing into a water bath set at 70 °C. The deionized water (H_2O) was purified by a purification system from ELGA PURELAB Ultra, Labotec. The extraction mixture was vortex mixed after five min and again at ten min when tubes were removed from water bath. After cooling at room temperature with the stopper off for an additional five min, the extracts were centrifuged at 2000*g* for ten min, with the resultant supernatant decanted into a ten ml measuring cylinder. The extraction step was repeated twice. Both extracts were pooled, and the volume adjusted to ten ml with cold 70:30 methanol: water (v/v).

A volume of one ml of the extract was diluted with water to 100 ml. The total polyphenol content (TPC) was determined according to ISO 14502-1: 2005 procedure, with Gallic acid (Sigma-Aldrich, South Africa) as standard. From the 1/100 ml extract sample dilution, a one ml volume was transferred in duplicate into separate glass tubes. Five ml of a 1/10 dilution of Folin-Ciocalteu reagent (Merck Chemicals, South Africa) in water was pipetted into each tube and mixed. After five min, four ml of anhydrous sodium carbonate (Sigma-Aldrich, South Africa) solution (7.5% w/v) was added to each tube, stoppered and mixed before being allowed to stand at room temperature for 60 min. The absorbance was measured at 765 nm on a Thermo Multiskan Ascent microplate reader against water. Gallic acid standards were used for quantification and the results were expressed as% Gallic acid equivalents (GAE) in g/100 g dry weight plant material. The Gallic acid standard curve which was linear from 10 to 50 μg/ml in the assay was used to measure the polyphenol content in each of the samples. TPC, expressed as a% (w/w) by mass on a sample dry matter basis, is given by the formula:

$$\%TPC = (OD_{sample} - OD_{intercept}) \times V \times d \times 100$$

$$Slope_{std} \times M_{sample} \times 10000 \times DM$$

where OD_{sample} is optical density obtained for the sample, $OD_{intercept}$ is optical density at the point the best fit linear regres-