Plant Science 246 (2016) 112–118

Contents lists available at ScienceDirect

Plant Science

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

Galactinol as marker for seed longevity

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 22 February 2016
Accepted 22 February 2016
Available online 27 February 2016

Keywords:
Arabidopsis
Galactinol
Galactinol synthase
Seed deterioration
Seed longevity
Raffinose oligosaccharide family

ABSTRACT

Reduced seed longevity or storability is a major problem in seed storage and contributes to increased costs in crop production. Here we investigated whether seed galactinol contents could be predictive for seed storability behavior in Arabidopsis, cabbage and tomato. The analyses revealed a positive correlation between galactinol content and seed longevity in the three species tested, which indicates that this correlation is conserved in the Brassicaceae and beyond. Quantitative trait loci (QTL) mapping in tomato revealed a co-locating QTL for galactinol content and seed longevity on chromosome 2. A candidate for this QTL is the *GALACTINOL SYNTHASE* gene (Solyc02g084980.2.1) that is located in the QTL interval. GALACTINOL SYNTHASE is a key enzyme of the raffinose family oligosaccharide (RFO) pathway. To investigate the role of enzymes in the RFO pathway in more detail, we applied a reverse genetics approach using T-DNA knock-out lines in genes encoding enzymes of this pathway (*GALACTINOL SYNTHASE 1*, *GALACTINOL SYNTHASE 2*, *RAFFINOSE SYNTHASE*, *STACHYOSE SYNTHASE* and *ALPHA-GALACTOSIDASE*) and overexpressors of the cucumber *GALACTINOL SYNTHASE 2* gene in Arabidopsis. The *galactinol synthase 2* mutant and the *galactinol synthase 1 galactinol synthase 2* double mutant contained the lowest seed galactinol content which coincided with lower seed longevity. These results show that galactinol content of mature dry seed can be used as a biomarker for seed longevity in Brassicaceae and tomato.

1. Introduction

Orthodox seeds can be stored dry after they have been dispersed by the mother plant. This ability is induced during seed maturation when profound physical, physiological and biochemical changes occur [1]. The final stage of the maturation process is marked by dehydration, in such a manner that during the reserve deposition phase, there is an accumulation of potentially protective molecules, especially soluble sugars, such as sucrose, raffinose and stachyose [2–4] and *LEA* (late embryogenesis abundant) proteins [5]. The *LEA* proteins act in synergism with the soluble sugars during cytoplasm crystallization and prevent damage caused by water removal from the seed tissues, by protecting the membrane surface [5–7].

Seed longevity represents the length of time that the seeds remain viable after attainment of physiological maturity [8]. Especially during dry storage seeds lose quality due to deterioration processes, which are inevitable and irreversible. The first indicator of deterioration is a reduction of the rate of germination (which is a vigour indicator) and, consequently, at a later period of storage, the loss of germinability [1,9]. The rate of the deterioration process is influenced by several factors, both during seed maturation when the maternal environment can influence the magnitude of longevity, and during drying and storage [10,11]. High light during seed maturation may notably increase seed longevity while low temperature decreases it in *Arabidopsis thaliana* (Arabidopsis) [12]. Moreover, high temperature and high relative humidity during storage, decrease seed viability and vigour significantly in several species [13,14]. Seed longevity is a quantitative trait and the behavior of longevity is species- or variety specific [10,15]. In order to study seed longevity in a short period of time, artificial aging tests were established in which seeds are exposed to high temperature and high relative humidity for a short period after which germinability of the seeds is analysed [16,17]. For Arabidopsis this method has been shown to mimic natural dry aging quite well [18,19].

Sugars in seeds have been proposed to act as signals that regulate and influence seed development [20]. Among these sugars are the raffinose family oligosaccharides (RFO, i.e. raffinose, stachyose

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http://dx.doi.org/10.1016/j.plantsci.2016.02.015
0168-9452/

and verbascose) for which a role in seed longevity has been suggested. Several studies have shown that sugars help to maintain the structural integrity of membranes and proteins under dry conditions due to the formation of a glassy state to limit deteriorative reactions [21–23]. Alternatively, the ratio of oligosaccharides to sucrose may be used as an indicator of seed quality and storability [7,9,21,24,25]. However, it has also been reported that the RFO are not a good indicator of seed vigour [19,24] or that it is not equally applicable in all species [26]. So far there is no definitive proof that RFO play a role in seed longevity.

The aim of this study is to investigate whether galactinol can serve as a marker for seed longevity. The use of a metabolic marker as indicator of seed longevity will facilitate the seed industry in making decisions on which seed lots can be placed onto the market or can be stored for longer periods of time. Galactinol is produced during the first step of the RFO biosynthesis pathway where UDP-galactose is converted to galactinol by GALACTINOL SYNTHASE (GOLS), which plays a regulatory role in this pathway [4,22,23]. Galactinol content is analysed in different Arabidopsis, cabbage and tomato genotypes and the revealed correlation with seed longevity is analysed in more detail using QTL mapping in tomato and T-DNA knock-out lines of genes encoding enzymes of the RFO pathway and overexpressors of the cucumber *GALACTINOL SYNTHASE 2* gene in Arabidopsis.

2. Materials and methods

2.1. Plant materials and growth conditions

2.1.1. Arabidopsis

Landsberg *erecta* (L*er*-0, L*er*), Columbia (Col-0, Col), *dog1-1* mutant [27] (with a L*er* genetic background) and *dog1-3* (SALK 000867, T-DNA insertion in the promoter region of *DOG1*, with a Col genetic background) were retrieved as described before [28]. T-DNA knock-out lines of genes encoding enzymes of the RFO biosynthetic pathway *galactinol synthase 1* (*gols1*) (AT2G47180—SALK_046018), *galactinol synthase 2* (*gols2*) (AT1G56600—SALK_101144), *raffinose synthase* (*rs*) (AT5G40390—SALK_085989), *stachyose synthase* (*sts*) (AT4G01970—SALK_045237) and *alpha-galactosidase* (*agal*) (AT5G08380—SALK_079578) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and the Arabidopsis overexpression lines of *GALACTINOL SYNTHASE 2* of cucumber (CsG26, CsG32 and CsG58 in Col-0) were obtained from Rainer Höfgen [29].

The plants were grown in a growth chamber (at 20 °C/18 °C (day/night) under a 16-h photoperiod of artificial light (150 μmol m^{-2} s^{-1}) and 70% relative humidity), using Rockwool blocks (4 × 4 cm) watered with Hyponex solution, in randomized complete block design with three or four replicates per genotype. The T-DNA knock-out lines were screened for homozygous insertions. Homozygous mutants of *gols1* and *gols2* were crossed in order to obtain the double mutant.

2.1.2. Cabbage

Six batches of proprietary cabbage seeds of three different varieties (Savoy Cabbage 1.1 and 1.2, Chinese Cabbage 2.1 and 2.2 and White Cabbage 3.1 and 3.2) were obtained from Bejo Seeds BV, The Netherlands. These seeds were produced in greenhouse conditions at BEJO seeds. The seeds were dried and size graded and subsequently stored at 20 °C in paper bags without control of relative humidity. Samples were taken at different intervals during storage over a period of 8 years, and then stored at −25 °C in aluminium bags until use.

2.1.3. Tomato

50 recombinant inbred lines (RIL) of tomato from a cross between *Solanum lycopersicum* cv. Moneymaker and *Solanum pimpinellifolium* were used. The tomato seeds came from the same lots as described by Kazmi et al. [30]. In short, plants were grown under greenhouse conditions at 25 °C/15 °C (day/night) and long day conditions (16 h light and 8 h dark). After harvest the seeds were processed and treated with trisodium phosphate ($Na_3PO_4.12H_2O$) for disinfection. Seeds were dried for 3 days at 20 °C and stored at 13 °C and 30% relative humidity in paper bags.

2.2. Seed longevity analyses

To measure seed longevity, an artificial aging test was executed by incubating seeds in open tubes over a saturated $ZnSO_4$ solution (40 °C, 85% relative humidity) [31] for 8 days for Arabidopsis and 15 days for tomato, according to preliminary tests (data not shown). Thereafter a germination assay was performed using two layers of blue germination paper equilibrated with 48 ml demineralized water in plastic trays (15 × 21 cm). Six samples of approximately 50–150 seeds were spread on wetted papers using a mask to ensure accurate spacing. Piled up trays were wrapped in a closed transparent plastic bag. The experiment was carried out in a 22 °C incubator under continuous light (143 μmol m^{-2} s^{-1}). Photographs were taken twice a day for a period of 6 days and analysed by the Germinator package [32]. For cabbage, natural aging (storage at 20 °C in paper bags without humidity control) was monitored by assessing the maximum germination percentage (Gmax) at several time intervals over an eight-year period.

2.3. Analysis of seed metabolites using gas chromatography with time-of-flight mass spectrometry (GC-TOF-MS)

Approximately 10 mg (Arabidopsis) or 30 mg (tomato) of dry seeds, pre-cooled in liquid nitrogen, were homogenized in 2-ml tubes with 2 iron balls (2.5 mm) using a dismembrator (Mo Bio Laboratory, MM 400). 375 μl methanol/chloroform (4:3) (Arabidopsis) or 700 μl methanol/chloroform (4:3) (tomato) and the standard ribitol (1 mg/ml) were added and mixed thoroughly. After 10 min of sonication (Ultrasonic Branson 3510), 100 μl (Arabidopsis) or 200 μl (tomato) of MQ water was added to the mixture followed by vortexing and centrifugation (5 min., 15,000 rpm). The methanol phase was collected in a new 2 ml Eppendorf tube. 250 μl (Arabidopsis) or 500 μl (tomato) of methanol/chloroform (1:1) was added to the remaining organic phase and kept on ice for 10 min. 100 μl (Arabidopsis) or 200 μl (tomato) of MQ water was added followed by vortexing and centrifugation (5 min., 15,000 rpm). Again, the methanol phase was collected and mixed with the other collected phase. 50 μl (Arabidopsis) or 100 μl (tomato) of the extract was dried overnight in a glass vial using a speedvac (room temperature, Savant SPD121P).

A GC-TOF-MS based method was used, as described in Carreno-Quintero et al. [33] with some minor modifications. Detector voltage was set at 1650 V. Raw data was processed using the chromaTOF software 2.0 (Leco instruments) and further processes using the Metalign software [34], to extract and align the mass signals. A signal to noise ratio of 2 was used. The output was further processed by the Metalign Output Transformer (METOT; Plant Research Internation, Wageningen). Centrotypes were created using the MSclust program [35]. The mass spectra of these centrotypes were used for the identification by matching to an in-house constructed library and the NIST05 (National Institute of Standards and Technology; http://www.nist.gov/srd) libraries. This identification is based on spectra similarity and comparison of retention indices calculated by using a 3th order polynomial function [36].