Plant Science 270 (2018) 1–12

Contents lists available at ScienceDirect

Plant Science

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

Phytic acid transport in *Phaseolus vulgaris*: A new *low phytic acid* mutant in the *PvMRP1* gene and study of the *PvMRPs* promoters in two different plant systems

Eleonora Cominelli[a,*], Massimo Confalonieri[b], Martina Carlessi[a,c], Gaia Cortinovis[a], Maria Gloria Daminati[a], Timothy G. Porch[d], Alessia Losa[a,e], Francesca Sparvoli[a]

[a] CNR – National Research Council, Institute of Agricultural Biology and Biotechnology (IBBA, CNR), Via E. Bassini, 15, 20133, Milan, Italy
[b] CREA Research Centre for Animal Production and Aquaculture (CREA-ZA), Viale Piacenza 29, 26900, Lodi, Italy
[c] Present address: Plantlab, Institute of Life Sciences, Scuola Superiore Sant'Anna, Via G. Guidiccioni, 8-10, 56010 Ghezzano (Pisa), Italy
[d] USDA-ARS, Tropical Agriculture Research Station, 2200 P.A. Campos Avenue, Suite 201, Mayaguez, 00680, Puerto Rico
[e] CREA Research Centre for Genomics and Bioinformatics (CREA-GB), Via Paullese 28, 26836 Montanaso Lombardo, Lodi, Italy

ARTICLE INFO

Keywords:
Phytic acid
low phytic acid (*lpa*)
Multidrug resistance protein (MRP) type ATP-binding cassette (ABC) transporter
Phaseolus vulgaris

ABSTRACT

Phytic acid ($InsP_6$) is the main storage form of phosphate in seeds. In the plant it plays an important role in response to environmental stress and hormonal changes. $InsP_6$ is a strong chelator of cations, reducing the bioavailability of essential minerals in the diet. Only a common bean *low phytic acid* (*lpa1*) mutant, affected in the *PvMRP1* gene, coding for a putative tonoplastic phytic acid transporter, was described so far. This mutant is devoid of negative pleiotropic effects normally characterising *lpa* mutants. With the aim of isolating new common bean *lpa* mutants, an ethyl methane sulfonate mutagenized population was screened, resulting in the identification of an additional *lpa1* allele. Other putative *lpa* lines were also isolated. The *PvMRP2* gene is probably able to complement the phenotype of mutants affected in the *PvMRP1* gene in tissues other than the seed. Only the *PvMRP1* gene is expressed at appreciable levels in cotyledons. *Arabidopsis thaliana* and *Medicago truncatula* transgenic plants harbouring 1.5 kb portions of the intergenic 5′ sequences of both *PvMRP* genes, fused upstream of the *GUS* reporter, were generated. GUS activity in different organs suggests a refined, species-specific mechanisms of regulation of gene expression for these two *PvMRP* genes.

1. Introduction

In the seed, up to 85% of total phosphorus is stored as phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate; $InsP_6$). During germination, the activity of phytases remobilizes the phosphorus stored as $InsP_6$ to support seedling growth. As $InsP_6$ is highly negatively charged at physiological pH, it binds mineral cations and easily precipitates in the form of phytate salts. Humans and non-ruminants digest $InsP_6$ poorly because phytases are not present in their digestive tract. Consequently, the presence of $InsP_6$ limits mineral and phosphorus bioavailability and this compound is considered an anti-nutritional factor as well as a major problem for water eutrophication due to its presence in animal manures [1].

Recently it has become clear that $InsP_6$, and its precursors and pirophosphorylated forms, derived from its metabolism ($InsP_7$, $InsP_8$), play a key role in different plant cell processes, such as the regulation of hormone activity [2–4,5], abiotic and biotic stress response [6,7], calcium and sugar signalling [8,9], phosphorus homeostasis [10,11], photomorphogenesis [12], chromatin modification and remodeling [13] and mRNA nuclear export [14].

Two different $InsP_6$ biosynthetic pathways can be distinguished: 1) the lipid-independent one, predominant in the seeds, consisting in the sequential phosphorylation of *myo*-inositol (Ins) ring and soluble inositol phosphates ($InsP_s$); and 2) the lipid-dependent one, mainly active in vegetative tissues, where phosphatidylinositol (PtdIns) and PtdIns phosphates are the substrates of phosphorylation [15]. In both routes, an early or substrate supply phase and an early-intermediate phase that generate $InsP_3$ are present, then the two pathways converge in a late

Abbreviations: *lpa*, low phytic acid; Ins, *myo*-inositol; $InsP_s$, inositol phosphates; $InsP_6$, phytic acid; PtdIns, phosphatidylinositol; MRP, multidrug resistance protein; ABC, ATP-binding cassette; EMS, ethyl methane sulfonate; GUS, β-glucuronidase; P, phosphorous; P_i, inorganic phosphorous; HIP, high inorganic phosphorous; PAP, phytic acid phosphorous

* Corresponding author at: CNR – National Research Council, Institute of Agricultural Biology and Biotechnology (IBBA, CNR), Via E. Bassini, 15, 20133, Milan, Italy.
E-mail addresses: cominelli@ibba.cnr.it (E. Cominelli), massimo.confalonieri@crea.gov.it (M. Confalonieri), m.carlessi@santannapisa.it (M. Carlessi), gaia.cortinovis@studenti.unimi.it (G. Cortinovis), daminati@ibba.cnr.it (M.G. Daminati), Timothy.Porch@ARS.USDA.GOV (T.G. Porch), alessia.losa@crea.gov.it (A. Losa), sparvoli@ibba.cnr.it (F. Sparvoli).

https://doi.org/10.1016/j.plantsci.2018.02.003
Received 22 December 2017; Received in revised form 2 February 2018; Accepted 5 February 2018
Available online 07 February 2018
0168-9452/

phase which converts $InsP_3$ to $InsP_6$ [1,16].

$InsP_6$ is stored in the form of globoids inside the storage vacuoles, where it is transported by different $InsP_6$ transporters, multidrug-resistance-associated proteins (MRP). MRP belong to the ABCC cluster of plant ATP-binding cassette (ABC) transporters that have been isolated and characterized in different species, as recently reviewed [17]. $InsP_6$ distribution in the seed depends on the species: in barley, wheat and rice 80% of $InsP_6$ is stored in the aleurone and bran (maternal teguments) and only a limited amount accumulates in the embryo. In maize, a similar amount is accumulated in the embryo and scutellum [18], while in legumes more than 95% of $InsP_6$ is present in the cotyledons [19].

The elimination or reduction of $InsP_6$ from seeds has been a subject of debate [20] because different in vitro studies have shown that this compound is a broad-spectrum antineoplastic agent [21]. However, the reduction of $InsP_6$ in cereal and legume seeds is considered an important goal for breeding in order to increase the bioavailability of micronutrients and improve seed nutritional quality, mainly to the benefit of populations in the developing world who subsist on diets based on these crops [22]. In the last two decades, a number of *low phytic acid* (*lpa*) seed mutants have been isolated in different species, through the use of forward and reverse genetics approaches, as recently reviewed [23]. Mutants affected in enzymes of the early and intermediate phases of the pathway show a decrease in $InsP_6$ content and a molar increase in free inorganic phosphate levels. Mutants affected in genes involved in the late steps of the pathway have decreased $InsP_6$ content, a small increase in free phosphate and increase of lower $InsP_s$ (inositol phosphates with up to five phosphate residues) content [15]. Moreover, *lpa* mutants that have been affected in transporter genes show a similar phenotype to mutants in enzymes of the first part of the pathway. Thus far *lpa* mutants that have been isolated and characterized fall into two groups and include: mutants in genes coding for ABC transporters, orthologous to the Arabidopsis *AtMRP5* gene [24], described in maize [25], rice [26], wheat [27], soybean [28] and common bean [29]; and mutants in putative sulphate transporters, described in barley and rice [30,31], affecting orthologs of the Arabidopsis AtSULTR3;3 protein [32].

Unfortunately, negative pleiotropic effects, such as low germination rates, reduced seed development and weight, and stunted vegetative growth were described for the majority of the studied *lpa* mutants due to the important role of $InsP_6$ in different physiological and developmental processes. This aspect may strongly limit the efficacy of the introgression of the *lpa* trait into breeding programs [15].

Common bean (*Phaseolus vulgaris* L.), the most consumed legume worldwide, is a major source of proteins and health-beneficial nutrients [33]. Although common bean seeds have a high mineral content, severe iron and zinc deficiencies, causing stunted growth, decreased immune function and anaemia, are quite common in countries where beans are a prevalent component of the diet. For this reason, common bean was chosen by the Harvest Plus programme, an initiative of the Consultative Group for International Agricultural Research (CGIAR), as one of the target species for iron biofortification [34,35].

In common bean, only the *lpa1* mutant, affecting the *PvMRP1* gene that codes for an ABC transporter has been reported thus far [29,36,37]. The *lpa1* seeds show a 90% reduction of $InsP_6$ content, a decrease of raffinose containing sugars by 25% and of *myo*-inositol by 30%, and a seven times increase of free iron [29,36]. In a study on volunteers, it was shown that iron absorption derived from the ingestion of *lpa1* beans is significantly higher than iron absorption from beans with normal $InsP_6$ levels [38], confirming that *lpa1* seeds are really biofortified. Moreover, the *lpa1* plants do not show the negative pleiotropic effects observed in mutants of other species affected in *PvMRP1* orthologs [17]. The presence in common bean of *PvMRP2*, a *PvMRP1* paralog, that is most likely able to complement the absence of a functional PvMRP1 transporter in all the organs but in the seed, may explain the absence of negative pleiotropic effects [29] such as reduced germination and stunted growth, described in other species where only one protein of this class is present [17]. Hence, the common bean system may elucidate the possible functional redundancy of the different paralogs, present also in other legumes such as soybean [28] and *M. truncatula* (sequences from Phytozome v12 website).

In the present study, we isolated a new *lpa* mutant and some other putative mutants from the screening of an ethyl methane sulfonate (EMS) common bean population [39]. The new *lpa* mutant is allelic to the *lpa1* previously described [29]. Quantitative expression analyses confirmed the hypothesis of functional redundancy of *PvMRP1* and *PvMRP2* in almost all plant organs with the exception of developing cotyledons, where only *PvMRP1* is expressed at appreciable levels. Moreover, a detailed analysis of GUS activity in *Arabidopsis thaliana* and *Medicago truncatula* transgenic plants harbouring the 5′ intergenic sequences upstream of the coding sequence of the *PvMRP1* and P*vMRP2* genes. These constructs were fused upstream of the *GUS* reporter gene and in silico analysis of these sequences revealed some differences between the two model species in the activity of these regulatory sequences, suggesting species-specific regulation of these genes.

2. Materials and methods

2.1. Plant material and growth conditions

The following genotypes of *P. vulgaris* were used:

- BAT 93 wildtype genotype developed at the International Center for Tropical Agriculture (CIAT, Cali, Colombia) and derived from a double cross involving four Mesoamerican genotypes that was kindly provided by CIAT;
- 1688 M_4 lines of the mutant population developed by Porch and colleagues (2009) in the BAT 93 background; these seeds were bulk harvested from plants grown in the field in Puerto Rico and Colombia;
- 905 F3 lines from a common bean breeding population described by Campion and colleagues [36];
- *lpa1* mutant described by Campion et al. [36] affecting the *PvMRP1* gene [29]. The mutation was introgressed in the BAT 93 background through three cycles of backcrosses to the recurrent parent, BAT 93. In each cycle BAT 93 was used as the female parent, and the selection of the *lpa* mutant seeds was performed in F_2 seeds using the conservative HIP assay (see below).

Common bean plants were grown in phytotron under short day conditions (10 h light at 26 °C, 14 h dark at 21 °C), at a relative humidity of 50%.

RNA was extracted from different organs collected at different developmental stages of seedlings or plants in the BAT 93 wildtype. Samples were collected from at least three different plants and pooled. Seeds were germinated on germination paper at room temperature in the dark for 2 days to collect root tips, or for 4 days in other cases. To collect young leaves at the 3rd trifoliate stage, shoot apical meristems, roots and nodules, seedlings were transplanted and grown for 3 weeks on a mixture of sand/agriperlite (3:1) in pots. The pots were watered every other day with the plant nutrient solution as reported by [40]. In order to collect flowers, pods, cotyledons and embryonic axes other seedlings were transferred directly to soil in pots. Entire pods were collected at 7 days After Flowering (DAF). Cotyledons and embryonic axes were collected at 14, 21 and 28 DAF. All samples were collected in triplicates and immediately frozen in liquid nitrogen and stored at −80 °C until use.

Arabidopsis thaliana Columbia (Col-0) ecotype plants were grown under long-day conditions (14 h light/10 h dark at 100 μmol m^{-2} s^{-1}) at 22 °C in a growth chamber either in Petri dishes or on soil. Transgenic lines were selected on Petri dishes, containing MS medium [41] (Duchefa Biochemie, Haarlem, The Netherlands), 1% w/v sucrose,