THE EXPRESSION PROFILES OF SELECTED GENES IN DIFFERENT BEAN SPECIES (*PHASEOLUS* SPP.) AS RESPONSE TO WATER DEFICIT

PROFILI IZRAŽANJA IZBRANIH GENOV V RAZLIČNIH VRSTAH FIŽOLA (*PHASEOLUS* SPP.) PRI ODZIVU NA POMANJKANJE VODE

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ABSTRACT

The aim of this study was to compare expression profiles of a number of transcripts from leaves of different *Phaseolus* species under drought stress, in order to ascertain whether changes in their expression in *Phaseolus* spp. are part of a general or a species specific response to drought. Relative gene expression analysis using quantitative PCR were carried out in *P. coccineus*, *P. lunatus* and *P. acutifolius* for 13 transcripts previously identified as up- or down-regulated in leaves of *P. vulgaris*. The mode of expression was found consistent within *Phaseolus* spp., despite the fact that the four species differ in their responses to drought at the physiological and morphological levels. The present results suggest that this is a common feature of the response of *Phaseolus* spp. The majority of the genes shown here to be influenced by water deficit in beans have been reported in other plant species under similar conditions, suggesting that they play a role in the general response to drought stress.

Keywords: Scarlet runner bean, Lima bean, Tepary bean, Water deprivation, Gene expression analysis, Quantitative PCR

IZVLEČEK

Primerjali smo profile izražanja nekaterih transkriptov v listih različnih vrst fižola, z namenom ugotoviti ali so spremembe v njihovem izražanju tekom pomanjkanja vode del splošnega odziva na sušo v rodu *Phaseolus* ali so specifične za posamezno

vrsto. Relativno analizo izražanja smo izvedli s kvantitativnim PCR pri vrstah *P. coccineus*, *P. lunatus* in *P. acutifolius* za 13 transkriptov, ki smo jih v prejšnji raziskavi pri *P. vulgaris* že potrdili kot navzgor ali navzdol regulirane. Pri vseh vrstah smo zabeležili podoben vzorec izražanja, ne glede na dejstvo, da se te štiri vrste različno odzivajo na sušo, tako na fiziološkem kot na morfološkem nivoju. Rezultati torej kažejo, da gre za del splošnega odziva v *Phaseolus* spp. Večina od tu analiziranih genov je udeležena v odziv na sušni stres tudi pri drugih vrstah rastlin, kar kaže, da igrajo vlogo v splošnem odzivu na sušni stres.

Ključne besede: turški fižol, limski fižol, ostrolistni fižol, pomanjkanje vode, analiza genske ekspresije; kvantitativni PCR

POVZETEK

Od 50-60 divjih vrst iz rodu *Phaseolus* je bilo udomačenih pet vrst: *P. vulgaris* L. (navadni fižol), *P. coccineus* L. (turški fižol), *P. acutifolius* A. Gray (ostrolistni fižol), *P. lunatus* L. (limski fižol) in *P. polyanthus* Greenm. [1]. Omenjene vrste imajo zelo različno toleranco na sušo, najbolj odporna na sušo je vrsta *P. acutifolius*. V predhodnih raziskavah smo v listih rastlin *P. vulgaris* identificirali 16 diferencialno izraženih transkriptov in pridobili profile izražanja tekom različnih stadijev suše [10, 11]. Namen te raziskave pa je bil pridobiti še informacije o njihovem izražanju pri treh drugih vrstah fižola, *P. coccineus*, *P. lunatus* in *P. acutifolius*. Za vrsti *P. coccineus* in *P. lunatus* smo pripravili dva poskusa (enega v rastni komori, drugega v rastlinjaku). Vsak poskus je vseboval normalno zalivane rastline (kontrolo) ter rastline v treh različnih stadijih suše (Pregl. 1). Zaradi pomanjkanja semen smo poskus za rastline *P. acutifolius* pripravili samo v rastni komori. RNA smo izolirali iz listov s pomočjo RNAgents Total RNA sistema za izolacijo (Promega, USA). cDNA smo sintetizirali s pomočjo začetnih oligonukleotidov (dT)15 in SuperScript II reverzne transkriptaze (Invitrogen, USA). Raven izražanja transkriptov smo ovrednotili s kvantitativnim PCR na podlagi SYBR safe tehnologije na 7500 Real Time PCR sistemu (Applied Biosystems, USA). Oligonukleotidni začetniki, ki smo jih uporabili za kvantitativni PCR, so navedeni v Pregl. 2. Za vse transkripte smo najprej preverili, če lahko uporabimo iste oligonukleotidne začetnike, kot smo jih uporabili za analizo vzorcev vrste *P. vulgaris*. Izkazalo se je, da je za kvantitativni PCR pri vrstah *P. coccineus*, *P. lunatus* in *P. acutifolius* uporabna večina, to je enajst od šestnajstih testiranih oligonukleotidnih začetnikov. Na vseh treh vrstah smo lahko uspešno pomnožili tudi referenčni gen (aktin). En transkript (DD5) se je uspešno pomnožil le pri *P. coccineus* in *P. acutifolius*. Za preostale transkripte smo poskušali poiskati homologne sekvence v javno dostopnih zbirkah EST zaporedij drugih vrst fižola (predvsem vrste *P. coccineus*) in nato na osnovi le-teh dizajnirati nove oligonukleotidne začetnike. Na ta način smo lahko analizirali še dva transkripta (CA1 in 25CA145). Rezultati so pokazali, da geni, ki so bili pri navadnem fižolu navzgor in navzdol regulirani, kažejo podoben trend tudi v vseh treh analiziranih vrstah fižola (Slika 1). Večina tu analiziranih genov je udeležena v odziv na sušni stres tudi pri drugih vrstah rastlin, kar kaže, da igrajo vlogo v splošnem odzivu na sušni stres. Študija je bila narejena na omejenem številu rastlin z namenom pridobiti osnovne informacije o odzivu

različnih vrst fižola na sušni stres, za pridobitev podrobnejših in natančnejših rezultatov o posameznih transkriptih pri določeni vrsti, pa bi bilo potrebno izvesti obširnejše študije.

INTRODUCTION

Beans (*Phaseolus* spp.) are the most important grain legumes for direct human consumption. Total production is almost twice that of chickpea (*Cicer arietinum*), which is the second most important grain legume [1]. Of the 50-60 wild *Phaseolus* species of American origin only five, namely common (*P. vulgaris* L.), yearlong (*P. polyanthus* Greenm.), scarlet runner (*P. coccineus* L.), tepary (*P. acutifolius* A. Gray), and lima (*P. lunatus* L.) bean have been domesticated [1].

In recent years, several studies have clarified the phylogenetic relationships between *Phaseolus* species. Studies on cpDNA [4, 5] and ITS sequences [6] have established a phylogeny for the entire genus. *P. vulgaris* is a part of a complex of species which includes *P. acutifolius*, *P. coccineus*, and *P. polyanthus*, while *P. lunatus* belongs to a separate group that includes the only South American radiation and oceanic island species of *Phaseolus*. Included are *P. mollis*, *P. pachyrrhizoides*, *P. augusti*, *P. bolivianus*, *P. viridis*, *P. lignosus*, and *P. lunatus* [6].

The group of *Phaseolus* species is remarkably diverse with respect to morphology (bushes to climbers, seed colour and colour patterns), adaptation (from hot deserts to cool mountain environments), and reproductive systems (from cleistogamy to out-crossing). Differences in drought tolerance between *Phaseolus* species have also been observed. The common bean is relatively sensitive to drought and heat stress [7]. The highest levels of drought tolerance are found in *P. acutifolius* which tolerates drought by postponing tissue dehydration through sensitive stomata [3, 13], an extensive root system [13, 18], greater water-use efficiency [3], and more active paraheliotropism [20]. However, *P. acutifolius* does not tolerate tissue dehydration more than *P. vulgaris* [2]. *P. acutifolius* had higher net photosynthetic rates than *P. vulgaris* at high to moderately low leaf water potentials [3]; however, stomatal conductance and net photosynthetic rate decreased more rapidly with decreased leaf water potential in *P. acutifolius* than in *P. vulgaris*. This response was related to increased stomatal closure. Furthermore, higher net photosynthetic rates, at any given internal CO2 partial pressure, led to higher water-use efficiency in *P. acutifolius* than in *P. vulgaris*.

There is increasing interest in genomic responses of *Phaseolus* species to drought stress. Torres et al. identified 20 early and four late dehydration-responsive genes in *P. vulgaris* roots [19], while 18 transcripts displaying differential accumulation in response to drought were found in *P. vulgaris* leaves and roots [15]. In comparative transcript profiling in roots of *P. acutifolius* and *P. vulgaris* under water deficit stress, Micheletto et al. identified 488 drought responsive genes in the former and only 64 genes in the latter [14].

In our previous study, fifteen differentially expressed transcripts were identified in leaves of *P. vulgaris* at different levels of dehydration [11]. We also identified another up-regulated transcript in leaves of *P. vulgaris* - putative dehydration-responsive element-binding protein (DREB) [10].

The aim of this study was to compare expression profiles of a number of transcripts from leaves of different *Phaseolus* species under drought stress, in order to ascertain whether changes in their expression are species specific or part of a general response of genus *Phaseolus* to drought. Quantitative PCR (qPCR) expression profiles of genes shown in our previous work to be up- or down-regulated in leaves of *P. vulgaris* have been determined for *P. coccineus*, *P. lunatus* and *P. acutifolius* under different levels of dehydration.

MATERIALS AND METHODS

Plant materials

Two sets of *P. coccineus* and *P. lunatus* plants were grown, one in the growth chamber under the conditions described in Kavar et al. [11], and the other in the greenhouse as described in Hieng et al. [9]. Each set consisted of one control (well-watered) plant and three plants under different levels of dehydration: from D1 (first stage of water deprivation) to D3 (severe water deficit) (Table 1). For the analysis of *P. acutifolius* (var. *latifolius* Freeman; PHAS 8442/00 IPK Gatersleben), one control (well-watered) plant and two drought-stressed plants (D1 and D2), were grown under controlled conditions in the growth chamber. Control plants were watered daily, while watering of drought-stressed plants was stopped when plants were 16, 18 and 21

Table 1. Leaf water content (WC), plant age at sample collection and duration of water deprivation for drought-stressed plants (D1, D2 and D3).
Pregl. 1. Vsebnost vode v listih (WC), starost rastlin ob odvzemu vzorca, ter trajanje obdobja brez zalivanja za rastline, ki so rasle v pogojih suše (D1, D2 in D3).

		Growth chamber experiment			Greenhouse experiment		
Species	**Plant**	WC (%)	Plant age at collection (days)	Water deprivation (days)	WC (%)	Plant age at collection (days)	Water deprivation (days)
P. coccineus	Control	88.4	23		89.5	29	
	D1	90.5	22	4	85.6	29	8
	D2	90.9	23	5	85.3	32	11
	D3	85.7	26	8	82.2	35	14
P. lunatus	Control	89.1	23		84.6	29	
	D1	89.7	22	4	84.3	29	8
	D2	88.4	23	5	81.0	32	11
	D3	85.8	26	8	77.9	35	14
P. acutifolius	Control	93.3	23				
	D1	87.8	23	7			
	D2	82.9	31	13			

days old. The hydration state of leaves was defined by measuring their water content (WC).

Expression analysis using quantitative PCR

Total RNA was isolated using RNAgents Total RNA isolation System (Promega, USA) according to the manufacturer's instructions. 75 mg of fresh leaf was transferred to a 2 ml tube containing 900 µl of denaturing solution, snap frozen in liquid nitrogen and stored at –80°C. First-strand cDNA was synthesized in a 20 µl reaction mix using Oligo(dT)15 primer and SuperScript II reverse transcriptase (Invitrogen, USA). cDNA samples were checked for genomic DNA contamination by performing PCR using primers that span at least one intron of the genomic sequence.

The mRNA expression levels were evaluated in a SYBR® Green I assay using a 7500 Real Time PCR System (Applied Biosystems, USA). PCR amplifications were performed in a 20 µl reaction containing 2x Power SYBR Green Master Mix (Applied Biosystems, USA), 5 pmol of each primer and 4 µl cDNA. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, followed by a dissociation curve stage.

Table 2. A list of primers used in the present study for gene expression analysis.
Pregl. 2. Seznam oligonukleotidnih začetnikov, ki smo jih uporabili za analizo genske ekspresije.

Transcript [Genbank ID of nucleotide sequence used for designing primers]		**Primer sequences**
actin	[CV537379]	(tggccgtacaactggtattg, gctctgcagatgtggtgaaa)[a]
DREB	Dehydration-responsive element binding protein [CV541537]	(cgaggaatacggatgaggaa, tgacatgttcacggaatcgt)[c]
DD5	Group III late embryogenesis abundant (LEA) protein [EC997026]	(gaagccgtgaagcaaactct, aagtgatgctgcaaagaagtg)[a]
CG18	Abscisic stress ripening-like protein [EC997018]	(ctggtggatttgcctttcat, gaagccattcactcccaaaa)[a]
CA7	Aldehyde dehydrogenase family 7 member A1 [EC997039]	(gcaagattatgaagagggcttg, ctcaagagccaccagcctac)[a]
CA1	Ethylene-responsive transcription factor [EC997016 & CA896999]	(gcattttcgaggagtcagga, gatggaggagggaaattggt)[b]
CC3	Hydrolase, hydrolyzing O-glycosyl compounds, cell wall invertase [EC997036]	(tgaagggagagtttgcatca, acatgcaacggtgtcaaaaa)[a]
DD19	Putative ankyrin-kinase [EC997027]	(tgctcaaaacaaggatggtg,

		ggatgccacctgaacttgat)[a]
Sch-frg	Germin-like protein [EC997023]	(gccattgcttttgctgtttt, ctgccctagcttagccactg)[a]
25CA145	Photosystem I light-harvesting chlorophyll a/b-binding protein [EC997017 & CA901639]	(ggcttttgctgagttgaagg, gtaagcccaggcattgttgt)[b]
NV	Chlorophyll a/b-binding protein CP24 precursor [EC997024]	(gctgctgctccaaagaagtc, accatggaactccactccag)[a]
DD7	Conserved hypothetical protein [EC997014]	(tgcctcttgataaggcacaa, aattcattctctggcgttcg)[a]
CG03	Small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase [EC997022]	(ttggagcatggtttcgtgta, atgcactgcacttgacgaac)[a]
CG05	Ribulose 1,5-bisphosphate carboxylase large subunit [EC997037]	(ttggggttatccgctaagaa, atgcctttgatttcacctg)[a]

[a] Original primer sequences of the transcripts reported by Kavar et al. [11]
[b] Primers designed according to the *P. coccineus* sequences due to the failure of PCR amplification by original primer sequences
[c] Primers designed according to the *P. vulgaris* sequence, which is similar to the *DREB* genes [10]

The primers developed for *P. vulgaris* [10, 11] were used to analyse the transcripts: actin, DD5, CG18, CA7, CG20, CC3, DD19, Sch-frg, NV, DD7, CG03, CG05 and DREB (Table 2). For the remaining transcripts novel primers were designed using Primer3 software [16]: for CG10, primers were designed according to *P. coccineus* EST CA901797 encoding the putative dTDP-glucose 4-6-dehydratase; for CA1, primers were designed according to *P. coccineus* EST CA896999 encoding the ethylene-responsive element binding protein (EREBP3-like); for 25CA145, primers were designed according to *P. coccineus* EST CA901639 encoding LHCII type I chlorophyll a/b-binding protein; for DD8, primers were designed according to P. coccineus EST CA900205 encoding carbonic anhydrase – like protein.

Gene expression data (Ct values) were evaluated using the comparative ΔΔCt method [12] for each set of plants. REST 2005 software [8] was used to determine whether differences between drought-stressed and control samples were statistically significant. In all cases, actin was used as the reference gene.

RESULTS AND DISCUSSION

Primers used for qPCR

We first tested whether the primers used for PCR amplification of 16 drought-responsive transcripts in *P. vulgaris* [10, 11], could be used for amplification of homologous genes in *P. coccineus*, *P. lunatus* and *P. acutifolius*. Using these primers

we were able to amplify eleven transcripts, including actin as reference gene, in all three species, as already determined for *P. vulgaris*. One transcript (DD5) was successfully amplified in *P. coccineus* and *P. acutifolius*, but not in *P. lunatus*. PCR amplification of the remaining five transcripts failed in all three species.

We assumed that this failure was due to mis-priming rather than to the absence of the genes. We therefore designed novel primers for their analysis. Nucleotide sequences of *P. vulgaris* transcripts CG10 (EC997013), CA1 (EC997016), CG20 (EC997015), 25CA145 (EC997017) and DD8 (EC997025) were blasted against expressed sequence tag (EST) records of *Phaseolus* spp. in NCBI's dbEST (http://www.ncbi.nlm.nih.gov/) in order to find the most similar sequences for designing novel primers. Although 20,120 ESTs of *P. coccineus* and 751 ESTs of *P. acutifolius* are deposited in the database (31-10-2007), we were able to design useful primers for only two transcripts, CA1 (ethylene-responsive transcription factor) and 25CA145 (photosystem I light-harvesting chlorophyll a/b-binding protein). The most similar sequences were CA896999 and CA901639, both encoding relevant homolog genes in *P. coccineus* (Table 2). They were successfully amplified in all three species. For *P. vulgaris* transcript CG10 (encoding putative imbibition protein, raffinase synthetase), the most similar sequence (CA901797 of *P. coccineus*) encoded putative dTDP-glucose 4-6-dehydratase, which is not the relevant homolog. For *P. vulgaris* transcript DD8 (encoding putative carbonic anhydrase), a similar sequence was found (CA900205 of *P. coccineus*), but novel primers did not generate PCR products. For transcript CG20 (encoding putative pfkB-type carbohydrate kinase family protein in *P. vulgaris*), no significant matches were found.

In terms of sequence similarity of transcripts chosen for this study, *P. lunatus* differs most from the other three. This could reflect the established phylogenetic relationships for the four *Phaseolus* species, where *P. vulgaris*, *P. coccineus* and *P. acutifolius* are more closely related, and *P. lunatus* is the most distant [6]. Our results also indicate that many of the primers that can be developed for any of the *Phaseolus* spp. could be useful for cross-species PCR amplification and analysis of gene expression in this plant genus under different conditions.

Gene expression analysis

Generally, the results of gene expression analysis were in agreement with the results obtained for common bean [11]. In all three species, the same trends of response (up- or down-regulation under drought stress) were observed (Fig. 1). Usually, the highest response for all transcripts was detected in plants which were under the highest level of drought stress. The same pattern of expression during drought stress was detected in the sets of plants grown in growth chamber and greenhouse (Fig. 1). Differences between sets lay mainly in the absolute level of transcripts.

Six transcripts were up-regulated under drought stress in all three species, and one transcript in two species only, whether grown in growth chamber or in greenhouse (Fig. 1). Two, DREB and CA1, show the highest sequence similarity to transcription factors from ethylene-responsive transcription factor (ERF) family (AP2/EREBP

family). CA7 has been ascribed to the member A1 of aldehyde dehydrogenase family 7. CG18 shows the greatest similarity to proteins from the family of abscisic stress ripening-like proteins. CC3 is a putative enzyme hydrolyzing O-glycosyl compounds and DD19 a putative ankyrin-kinase. The up-regulation of transcript DD5, which can be assigned to the group III LEA proteins, was shown only for *P. coccineus* and *P. acutifolius*. This transcript could not be amplified in *P. lunatus*. As reported for *P. vulgaris*, all seven up-regulated transcripts are similar to genes shown to be influenced by drought in other plant species [11], although they differ from those reported by Montalvo-Hernandez et al. for common bean [15]. However, some of the latter belong to similar functional classes.

Six transcripts were down-regulated, again in all three species (Fig. 1). Transcript Sch-frg shows the highest similarity to genes for germin-like proteins (GLPs). The sequence of transcript DD7 is similar to that of the conserved hypothetical protein of *Medicago truncatula*. Two transcripts, 25CA145 and NV, exhibited the greatest similarity to the chlorophyll a/b-binding proteins (cab). CG03 has been ascribed to the small subunit and CG05 to the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). The small unit of Rubisco and *cab* genes were identified as being more than 5-fold repressed in *Arabidopsis thaliana* under drought stress [17]. As already pointed out for *P. vulgaris* [11], genes for GLPs, for the conserved hypothetical protein of *Medicago truncatula* and for the large unit of Rubisco have not previously been reported as being influenced by drought. The present study shows down-regulation of corresponding transcripts in the three other *Phaseolus* species, strengthening the conclusion that they are involved in response to water deficit in plants belonging to this genus.

As in *P. vulgaris* [11], the smallest response was detected in DD19, CC3 (both up-regulated) and CG05 (down-regulated) transcripts.

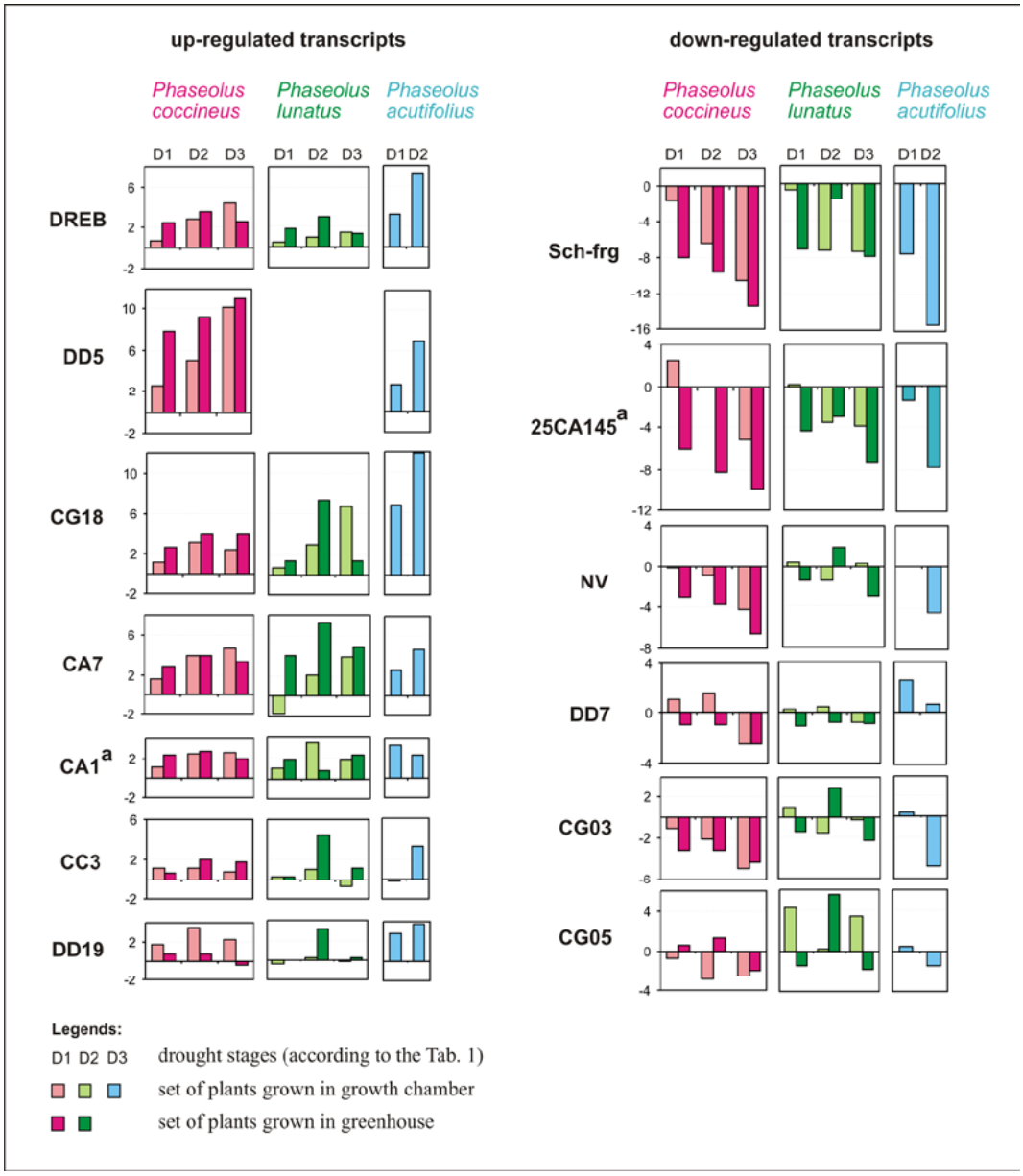
We already previously pointed out that the genes whose expression was changed under drought in leaves were different from those in roots, despite the fact that, in both organs, the corresponding transcripts showed significant similarity to well characterized plant genes [11]. This was not significantly changed by the recent comparative study on transcript profiling in roots of *P. acutifolius* and *P. vulgaris* under water deficit stress [14].

Differences within *Phaseolus* spp.

The differences in gene expression profiles between the three species were small and related only to the expression ratio (Fig. 1); the trend of response (up- or down-regulation under drought stress) being the same. Nevertheless, the response of *P. lunatus*, in terms of patterns of gene expression during drought stress for several transcripts (e.g. DREB, CG18, DD19, NV, DD7, CG03 and CG05), is less strong than that of the other two species. This could again be due to its greater genetic distance from other *Phaseolus* spp., already pointed out for the sequence similarity of transcripts chosen for this study. It might be reflected in the ability of *P. lunatus* to cope with drought. The response of *P. acutifolius* was the strongest (Fig. 1).

Fig. 1. Relative gene expression (-ΔΔCt) in leaves of plants of *P. coccineus*, *P. lunatus* and *P. acutifolius* for 14 transcripts. ΔΔCt was calculated by the equation: $\Delta\Delta Ct = (Ct_{target} - Ct_{actin})_{drought} - (Ct_{target} - Ct_{actin})_{control}$.

Slika 1. Relativna genska ekspresija (-ΔΔCt vrednosti) za 14 transkriptov v listih rastlin vrst *P. coccineus*, *P. lunatus* in *P. acutifolius.* ΔΔCt smo izračunali po enačbi $\Delta\Delta Ct = (Ct_{tarčni} - Ct_{aktin})_{suša} - (Ct_{tarčni} - Ct_{aktin})_{kontrola}$.



Our results for leaves of *P. vulgaris* and *P. acutifolius*, in which almost all the transcripts exhibited similar expression profiles, differed strikingly from those for roots [14], for which a much greater proportion of drought responsive genes was found in *P. acutifolius* (n=488) than in *P. vulgaris* (n=64). Only 25 genes were common to the two species. In the present study on leaves, all transcripts analysed responded to drought stress similarly, regardless of species or drought tolerance/susceptibility. These results could reflect the important role known at the morphological level for the root system of *P. acutifolius* in drought resistance.

CONCLUSIONS

Six genes were up-regulated in leaves of drought stressed plants of *P. lunatus*, *P. acutifolius*, *P. coccineus* and *P. vulgaris*, and one only in the last three. These genes encode enzymes such as protein kinases and aldehyde dehydrogenases, LEA proteins, osmoprotectants, transcription factors and cellular and carbohydrate metabolism. Six genes were down-regulated, the majority of which belong to the functional group of photosynthesis.

The fact that the mode of the expression of the studied genes is common to all four studied *Phaseolus* species indicates that these genes form part of a general and intrinsic response to drought of the genus *Phaseolus*, and not just of specific species.

Parallel studies on plants grown in growth chamber and in greenhouse experiments revealed the same trends of gene expression patterns in the response to drought. This strongly suggests that the same is true in the field, with clear application to breeding for drought resistance. However, this study was performed only on a small set of plants, just to provide basic information about the response to drought in each species examined. To provide more detailed (accurate results), further studies should be carried for each gene on different sets of plants.

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