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Z. G. AYTASHEVA *Green-house harvested ryegrass plants contain thioredoxin-like gene fragment in the pollen.* BIOTRONICS **27**, 53-60. Leaf genomic DNA, leaf and pollen poly (A+)-RNA preparations have been extracted from ryegrass, *Lolium perenne* L. grown under green-house conditions. A 790-nt fragment has been identified with a specific degenerative primer pair to the conserved 3'-terminal region of putative S-gene of *Phalaris coerulescens* L. using PCR of total DNA purified from self-incompatible mutants. A 214-nt amplification product has been detected using same specific primers in course of RT-PCR. Sizes of DNA and cDNA fragments generated appeared to be in a strict conformity with corresponding sequences earlier established for the pollen of other cereals, such as *Phalaris coerulescens* L. and *Secale cereale* L. Thioredoxin H-like sequence and its product is thus likely to be either essential for ryegrass self-incompatibility, moreover that multifunctional behaviour of the protein might also contribute to corporative interaction of tissue-unspecific, ubiquitous genes at flowering.

Key words: *Lolium perenne* L.; gametophytic self-incompatibility; pollen; S locus; thioredoxin.

INTRODUCTION

Gametophytic self-incompatibility (GSI), revealed in more than 15 families, was studied in four of them at the molecular level (*16*). A monofactorial GSI based on the action of a single, though multiallelic S locus was established to functiion in the Solanaceae, Scrophulariaceae and Rosaceae. Pistil-specific S locus of *Nicotiana alata* L. appeared reminding the SLG-SRK tandem typical for SSI system (*8*). Tobacco S locus exhibited conservative, clusterly-settled C1-C5 sites, interspersed by two variable regions. Despite relatively high degree of the S-locus divergence, referred to its polymorphism, major part of the solanaceous S proteins (22-34 kDa) displayed the ribonuclease activity and substantial RNase

Abbreviations : SI, self-incompatibility ; GSI, gametophytic self-incompatibility ; SSI, sporophytic self-incompatibility ; SLG, S-locus ghycoprotein ; SRK, S-locus receptor-protein kinase; RT-PCR, reverse transcription-polymerase chain reaction; CTAB, cetyl triethyl ammonium bromide ; DEPC, diethylpyrocarbonate.

sequence similarity to the amino acid sequences of fungal ribonucleases (i. g., from *Aspergillus oryzae* or RNase T2). Hence, S proteins are regarded as recognition molecules with intrinsic RNase activity (*9*). Bifactorial GSI, determined for cereals, is considered as the latest, second-evolving system, arising from initially monofactorial GSI system, that has intermediately developed in SSI (*19*). A two-factor GSI acts employing two unrelated multiallelic loci, S and Z (*4, 13*). S locus was shown to involve thioredoxinH-like sequence, expressing little enzymatic activity in self-fertile mutants (*13, 14*).

English ryegrass, *Lolium perenne* L. belongs to the genus *Poeae* of genuine grasses (*Poaceae*, subfamily *Pooideae*). Alike other three species, *L. multiflorum* Lam., *L. rigidum* Gaud. and *L. canariense* Steud., it is referred to as an interfertile and capable of outbreeding in various genetic combinations. Owing to its perennial nature, competative abilities and nutritional values, *L. perenne* has been estimated to constitute almost 50% of fodder varieties. On the other hand, moderate growing rates suggest its application as a lawn coverage (*4*). Passage across SI barriers, limiting closer genetic manipulations between ryegrass varieties, could have enabled breeders to develop offspring hybrids, which might simultaneously carry appropriate parental gene combinations and complex resistance. However, little is still known about GSI mechanisms in cereals. Likely GSI systems have been established for *Phalaris coerulescens* L. and *Secale cereale* L. (*4, 6, 13*). The purpose of this study was to analyse ryegrass genomic DNA and mRNA fragments for their similarity with the putative S-locus conserved 3'-terminus, previously examined in cereals forementioned.

MATERIALS AND METHODS

Genetic stocks

The plants of *L. perenne* (cv. Lilotta), used for the isolation of nucleic acids, represent part of a ryegrass collection held at the Federal Centre for Breeding Research on Cultivated Plants, Gross Luesewitz, Germany (*12*). Plants were previously mapped by the isoenzyme markers disposition in reference to the putative S locus (*4*). They were grown at room temperature in small pots and exposed to fluorescent lights for 12 hr daily for two weeks. Then leaves were cut, lyophilized and stored at −80℃ until DNA isolation. For RNA extraction leaves were collected on the day of isolation and ground using mortar and pestle with liquid nitrogen. Part of the collection was kept in green house until the ear formation, flowering and pollen maturation. Ears of each plant were wrapped in transparent cellophane envelopes 4-7 days before anther dehiscence, followed by mass pollen shedding, in order to collect pollen samples twice a day, keeping the pollen shortly on ice prior to storage at -80℃.

DNA isolation and amplification

Lyophilized leaves from separate SI plants were ground in a very fine powder and transferred to a Falcon tube. DNA extraction was performed in two volumes of hot CTAB by Saghai-Maroof *et al.* (*17*) with minor modifications.

DNA quality and quantity were asserted both spectrophotometrically and visually by ethidium bromide staining of agarose gels.

Sequences of upstream and downstream primers specific to *Phalaris coerulescens* domains V and VI of the 3'-terminal part of S locus (*13*) were:

TCC TGG TGT GGG CCA TGC CGT (No. 1, upstream, sense)
GGT GGC ACG GAT GTC CCA TGT (No. 2, downstream, anti-sense)
GAT CTG CTG GCC ATT CTT GAG (No. 3, downstream, antisense).

The PCR 20 μl reaction volume comprised 2 μl 10 × incubation buffer, 1 μl (2.5 mM each dNTP), 0.1 μl (0.5 U) Taq DNA Polymerase (Appligene-Oncor), 0.5 μl (5 μM) each primer and 10 μl (50 ng) leaf genomic DNA and redistilled water. The reaction was carried out in 35 cycles by the following temperature protocol: 95℃ for 1 min (1 × time); 95° for 1 min, 52° for 1 min, 72° for 45 sec (3 × times); 94° for 30 sec, 52° for 30 sec, 72° for 45 sec (32 × times); 4℃ (cooling).

RNA isolation and RT-PCR

Total leaf and pollen RNA was isolated using conventional phenol extraction by Scott (*18*). Poly (A+)-RNA was purified using Eppendorf Poly A+ Isolation Kit. RNA quantification and quality evaluation was achieved by estimation of the UV absorbance ratio at 260 and 280 nm.

Poly (A+)-RNA obtained was analized by RT-PCR according to Bell (*2*). 0.5 μg RNA in 9 μl of DEPC-treated water were pre-heated with 1 μl of the lower primer (10 μM, No. 3) at 65℃ for 5 min, then cooled on ice and added to the reverse-transcription reaction mixture (20 μl). It contained 6 μl 5 × RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl^2$), 3 μl 100 mM DTT, 1.5 μl RNasin (7.5 U of ribonuclease inhibitor from human placenta), 3 μl mixed dNTP stock (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), 2 μl (400 U) Superscript RNase H-Reverse Transcriptase (Gibco, BRL) and DEPC-treated water. Reaction was carried out at 45℃ for 60 min and stopped by addition of 1 μl 0.5M EDTA on ice. Subsequent PCR was performed using 1.5 μl of the RT cocktail containing cDNA. 2 μl 10 × PCR incubation buffer, 2 μl of each primer (5 μM), 1.5 μl dNTP (10mM each) and 0.1 μl (0.5 U) Taq DNA Polymerase were mixed in the reaction tube contaning 12.9 μl DEPC-treated water. 1.5 μl cold RT mixture were finally added to the tube. Temperature protocol was as follows: 95℃ for 2 min (1 × time); 94° for 30 sec, 60° for 30 sec, 72° for 45 sec (30 × times); 4℃ (cooling). PCR products were separated on 1.5% agarose gels. Control reactions included omission of one of two PCR primers. These reactions failed to amplify any detectable PCR products, indicating that the products are really specific amplified cDNA products, which do not contain contaminating genomic DNA in RNA preparation.

RESULTS AND DISCUSSION

It is generally accepted, that in *Phalaris coerulescens, Secale cereale* and other members of the *Poaceae* SI is accomplished due to a two-factor mechanism (*6,*

13). Putative S gene of *Phalaris* was established to contain on its 3'-terminus thioredoxin H-like sequence (*13*). Thus, Li *et al.* have already predicted, that most of the cereals with a chromosome formula N=7 × n should have conserved linkage groups, consisting of the S locus, similar to that one depicted for *Phalaris*, and a putative Z locus yet to be identified.

In this study genomic DNA extracted from leaves of individual ryegrass SI plants with known iso-enzyme gene marker disposition in reference to the putative S locus (*4*) was subject to PCR. At it is seen from Fig. 1 (lanes B and C), by using *Phalaris coerulescens*-specific primers, which correspond to the thioredoxin H-encoding 3'-terminus of the putative S locus (*13*), a single amplification product of ~790 nucleotides (application of primers 1 and 3, see Materials and Methods) or 740 nucleotides (primers 1 and 2) has been detected. When compared to the respective database of *Phalaris* (*13*), product sizes do correspond to the deduced *Phalaris* fragments to be generated under same PCR conditions. Relevant 700-nucleotide fragment was earlier shown for rye leaf

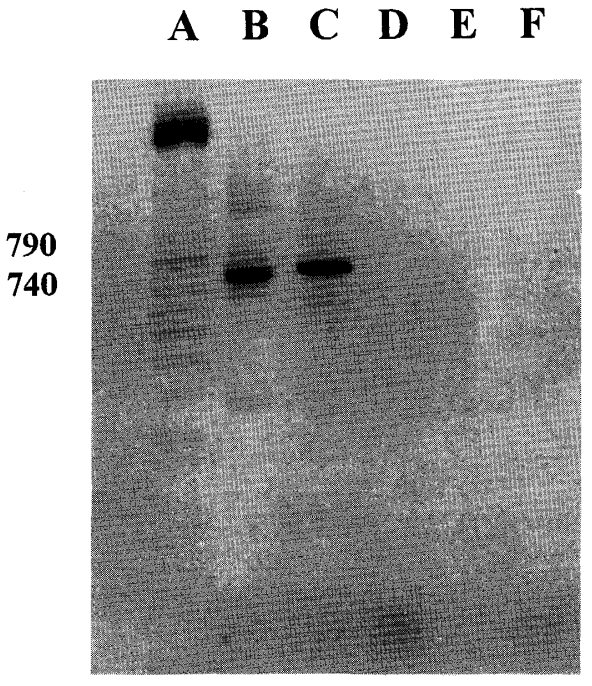

Fig. 1. Amplification profile of *L. perenne* genomic DNA with primers specific to thioredoxinH-like sequence of putative S locus of *Phalaris coerulescens* after 35 cycles.
(A) 100 bp DNA ladder (BRL);
(B) Amplification product (740 nt) using primers No. 1 (TCC TGG TGT GGG CCA TGC CGT) and No. 2 (GGT GGC ACG GAT GTC CCA TGT);
(C) Amplification product (790 nt) using primers No. 1 and No. 3 (GAT CTG CTG GCC ATT CTT GAG)
(D) Negative control (omission of primer No. 3);
(E) Negative control (omission of primer No. 2);
(F) Negative control (omission of primer No. 1).

DNA by using same primers derived from the S-locus exons V and VI (*6*). Rye fragment indicated an RFLP linked to the S locus with no recombination among 55 genotypes tested. Control panel (Fig. 1; lanes D, E and F) has displayed no amplification in the presence of one out of two primers as well, as under absence of primer constructs in the tube. Rye pollen thioredoxin-like 240-nucleotide cDNA amplification product (*6*) has been used as positive control (data not shown).

RT-PCR of poly (A+)-RNA from mature pollen of *L. perenne* SI mutants with the same upstream and down-stream *Phalaris coerulescens*-specific primers has shown a single, 240-nucleotide amplification product (Fig. 2). It has appeared to be identical to the 240-nucleotide cDNA sequence from rye pollen, pistils, ovaries and leaves, which were observed in the same reaction (*6*). Similar 240-nucleotide RT-PCR product has been obtained using ryegrass mRNA, purified from the pollen of different SI mutants and corresponding leaf samples (Fig. 3). As total RNA from *Phalaris* anthers shedding pollen was demonstrated to hybridize to the 907-nucleotide long Bm2 cDNA clone representing the S gene (*13*), mature ryegrass anthers could have either been handled instead of pollen batches to facilitate collecting the material, thereby rising its yield.

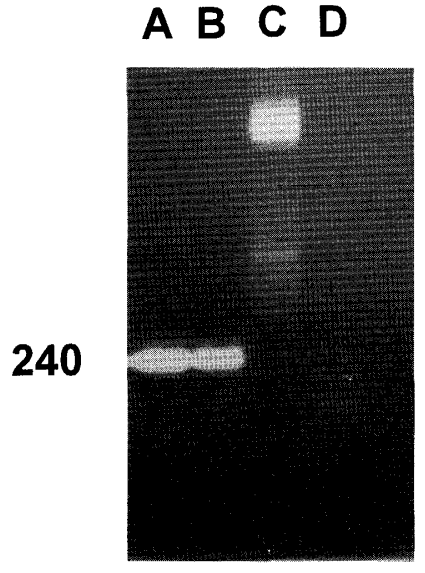

Fig. 2. The RT-PCR product (240 nt) of poly (A+)-RNA from the pollen of *L. perenne* using primers (Nos. 1 and 2, as in Fig. 1) specific to thioredoxinH-like sequence of the putative S locus of *Phalaris coerulescens* after 30 cycles. 20 μl PCR product was electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. Lanes:

(A) Positive control (PCR of pollen cDNA fragment from *Secale cereale* using same primers, see reference (6);
(B) RT-PCR of 0.5 μg pollen poly (A+)-RNA from the SI ryegrass plant;
(C) 100 bp DNA ladder (BRL);
(D) Negative control (omission of primers).

Previously deduced S-protein sequence of *Phalaris* was found to consist of 282 amino acid residues (Mr~31 kDa) and contain high percentage of Pro, Val and Lys (11.3, 17.8 and 5.7% respectively). Active site (-WCGPC-) is located in 《the avant-corps》 of conserved C-terminus, whereas the N-terminus is regarded as an allele-specificity region (*13*). It was proposed, that remarkable reduction of thioredoxin activity in a self-fertile *Phalaris* mutants might repress phosphorylation of the substituted amino acid residues, at the supreme moment abolishing SI rejection (*14*). Nevertheless, loss of SI response in result of decreased thioredoxin activity was recognized to be scarcely sufficient for sketching a general model of cereal GSI.

If SRKs and SLGs determined in *Brassica* are regarded as evolutionally advanced S-locus products, encoded by a multigene family in the genome of Cruciferae and some other angiosperms, cereal thioredoxin, deduced from corresponding database as S-locus product, along with the solanaceous S-RNases, is admittedly more ancient and present in both eukaryotic and prokaryotic cells (*5, 15*). Evidences on the diversity of SI products among families might reflect as overall protein accumulation in reproductive tissues, when compared to vegetative tissues, and enriched antigen representation of the male gametophyte, comparing to sporophytic tissues of the pistil (*10, 11*). On the other hand, eukaryotic origin of thioredoxin H might explain its eminent variability in comparison with other thioredoxin families. Interestingly, five complete thioredoxin H sequences were recorded for *Arabidopsis thaliana*, which are supposed to undergo divergence, preceding the subdivision between monocots and dicots (*7*).

Complementary to its presumable role in SI signalling, Bower *et al.* (*3*) proposed a more general biological function for thioredoxin H. It was detected

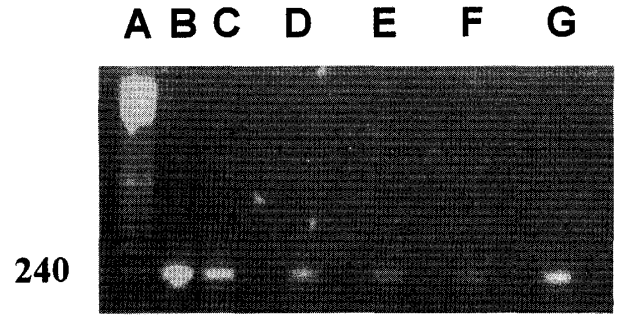

Fig. 3. The RT-PCR product (240 nt) of poly (A+) -RNA from leaves and pollen of various *L. perenne* plants using *Phalaris-specific* primers (Nos. 1 and 2, as in Fig. 1). Lanes:
(A) 100 bp DNA ladder (BRL);
(B) Positive control (PCR of pollen cDNA fragment from *Secale cereale* using same primers, see reference (*6*);
(C) RT-PCR of total ryegrass leaf mRNA;
(D) RT-PCR of leaf mRNA from an individual SI ryegrass plant;
(E), (F) and (G) RT-PCR of pollen mRNA from individual SI ryegrass plants.

that thioredoxin H-like cDNA, generated by RT-PCR from pistil poly (A+)-RNA of *Brassica napus*, able to specifically interact with the SRK domain. No interaction was however shown for related SRK domain of *Arabidopsis thaliana*. Noteworthy, in contrast to the SLG-SRK-tandemed S locus mediating SSI response, cereal S gene was clarified to encode soluble but not secreted thioredoxin-like protein in the pollen (*13*). As a major compound of the phloem sap, it seems very likely, that thioredoxin H serves as an ubiquitous plant signalling molecule, activating reduction-oxidation processes by relevant thioredoxin interactions with tissue-specific receptors (*7*). Hence, detailed relations of thioredoxin-like S-product to esterases, glycosyltransferases, phenolic compounds or pectin polysaccharids, induced in response to pollination, would be of a special interest, since GSI reaction happens, as a rule, early, on the stigma surface or in its hairspring, supposedly accumulating there a good number of unspecific molecules (*5*). Lastly, thioredoxin H association with bacteriophage T7 DNA polymerase was shown to accelerate DNA processivity (*1*). Serving as the processivity factor for the class I DNA polymerase, thioredoxin H is supposed to ensure viral genome replication. This finding might either update current knowledge on thioredoxin H activities at different stages of plant development.

Relatively modest progress in cereal SI mechanisms may be accounted for by especially rapid progamic stage of fertilization coupled to successive, therefore contradictory origin of a two-factor GSI system itself (*19*) : finely differentiated SI machinery, aimed at highly developed embryo, is set in motion by relatively primitive control mechanism with regard to angiosperm evolution. The discrepancy might be resolved upon elucidating thioredoxin H relationships discussed above.

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