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Identification of genes involved in a water stress response in timothy and mapping of orthologous loci in perennial ryegrass

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Abstract

In order to characterize the response of selected grasses to water stress, relative water content (RWC) in leaves and quantum efficiency of photosystem 2 (F_v/F_m) were measured in *Phleum pratense* L., *P. bertolonii* DC. and *P. phleoides* H. Karst. during 6 d of water stress. The results indicated differential responses to water stress among the three *Phleum* species with higher water deficit sensitivity of *P. pratense* and *P. bertolonii* than that of *P. phleoides.* The cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique was applied to identify differentially expressed genes responding to water stress in *P. pratense*. Cloned and sequenced differentially expressed fragments (DEFs) were used for primer design in order to identify orthologous genes in *Lolium perenne* L. Twelve genes orthologous to *P. pratense* DEFs were mapped in the *L. perenne* mapping population VrnA based on a high resolution melting curve analysis (HRM). This study provides genomic information about 29 differentially expressed genes after water stress in *P. pratense* and reports on the identification and mapping of twelve orthologs in *L. perenne*.

Additional key words: cDNA-AFLP, chlorophyll fluorescence, high resolution melting curve analysis, linkage mapping, *Lolium perenne*, *Phleum bertolonii, Phleum phleoides, Phleum pratense*, RWC.

Introduction

During the recent decades, the influence of anthropogenic and environmental factors on plant production became more relevant. Global warming is evident and according to applied scenarios (Meehl *et al.* 2007), the growing season in Europe is becoming warmer and dryer, especially in late summer and early autumn. Drought as well as other abiotic stresses such as high temperatures, oxidative stress, heavy metal pollution and salinity can severely impair plant performance. It significantly limits plant growth, development and crop productivity (Araus *et al.* 2002). To cope with abiotic stresses, plants have developed a wide spectrum of molecular mechanisms to sense changes rapidly and adapt accordingly (Chen *et al.* 2006, Kurek *et al.* 2007, Fernandes *et al.* 2008, Chae *et al.* 2009, Rodrìguez-Milla and Salinas 2009). Among the abiotic factors affecting plant growth, water availability is the most important one (Bray *et al.* 2000). This is due to the unpredictability of its occurrence, severity, timing and duration, as well as to the interaction of drought with other abiotic stresses (Ceccarelli and Grando 1996).

The plant defense response to water stress is associated with the synthesis of osmolytes, production of phytoalexins, activation of the general phenylpropanoid pathway and induction of lignin biosynthesis (Anami *et al.* 2009). Salicylic acid, methyl salicylate, jasmonic

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Abbreviations: cDNA-AFLP - complementary DNA - amplified fragment length polymorphism; DEF - differentially expressed fragment; EST-SSR - expressed sequence tag - simple sequence repeat; F_v/F_m - variable to maximum chlorophyll fluorescence (maximum potential quantum efficiency of photosystem 2); HRM - high resolution melting curve analysis; LG - linkage group; QTL - quantitative trait loci; RWC - relative water content.
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acid and methyl jasmonate can also serve as signaling molecules activating acclimation responses (Shulaev *et al.* 2008). Other substances such as ascorbic acid, glutathione, tocopherols, anthocyanins and carotenoids protect plants from oxidative stress (Tabaeizadeh 1998). Most of our knowledge on the molecular responses to water stress has been deducted from studies in the model plant *Arabidopsis*. However, the knowledge gained from model plants is not always directly transferable to the species of interest.

Breeding activities in forage species have mainly directed selection towards increasing biomass yield while natural selection has favoured mechanisms for adaptation and survival under different environmental conditions. Mechanisms to resist water stress in Boehmer's cat's-tail (*Phleum phleoides* (L.) H. Karst.) are coordinated by adjusting growth, development, cellular and molecular activities (Hubbard 1992, Židonytė 1992). Likewise, timothy (*Phleum pratense* L.), one of the most productive grass species in terms of first cut yield, forms low aftermath growth under dry conditions (Lemežienė *et al.* 2004). Timothy is valued for its winter hardiness, good palatability, and moderate nutritional feed value and thus is a major forage crop in northern latitudes. Its genomic constitution is complex (AAAABB) with the A-genome originating from the tetraploid form of *P. alpinum* and the B-genome coming from the diploid *P. bertolonii* (Cai and Bullen 1994). Unfortunately, the genetic and genomic tools available for this species are limited. *Phleum* spp. belong to the *Poaceae* family as do ryegrasses (*Lolium* spp.) and major crop species such as wheat, oat and rice. The genera *Lolium* and *Phleum* are believed to share common ancestors with seven chromosomes, *L. perenne* being diploid (2n=2x=14) and *P. pratense* having a hexaploid (2n=6x=42) set of chromosomes. Several studies established a comparative relationship between perennial ryegrass and other *Poaceae* genomes (Jones *et al.* 2002, Alm *et al.* 2003) and found that the genetic maps of perennial ryegrass and other closely related crops are conserved in terms of orthology and colinearity. Meanwhile, genome mapping data in *Lolium* spp. have been rapidly accumulated over the recent years (Studer *et al.* 2010). The construction of molecular marker-based genetic linkage maps using pseudo-testcross populations has provided the basis for trait-dissection in perennial ryegrass. For example, the VrnA F2 two-way pseudo-testcross population has been extensively characterised for various traits, such as vernalization response, disease resistance, seed yield and fertility traits (Jensen *et al.* 2005, Schejbel *et al.* 2007, 2008, Studer *et al.* 2008). To date, the genetic and genomic tools available for this mapping population include a moderate density genetic linkage map based on expressed sequence tag - simple sequence repeat (EST-SSR) markers (Studer *et al.* 2010), a large scale expressed sequence tags (ESTs) collection (Asp *et al*. unpublished data) and a PCR-screenable BAC library (Farrar *et al.* 2007), making VrnA a good model for studying the physiological, molecular and genetic basis of water stress in perennial forage grass species.

Whole genome technologies for differential gene expression analysis provide efficient tools to identify key genes that respond to water stress. Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis detects a large number of differentially expressed transcripts in a genome-wide range (Bachem *et al.* 1996) at low cost when compared to other techniques, such as microarray hybridisation, suppression subtractive hybridization (SSH) and transcriptome sequencing (RNA-seq). The cDNA-AFLP method does not require prior sequence information and was successfully applied to identify novel plant genes related to self-incompatibility, pathogen and drought response (Rechsteiner *et al.* 2006, Wang and Bughrara 2007, Kakeda *et al.* 2008, Van Daele *et al.* 2008).

Genetic mapping of differentially expressed fragments (DEFs) identified by cDNA-AFLP depends on sequence information and the development of genetic markers within DEFs. However, the efficiency of converting cDNA-AFLP fragments into sequence tagged sites (STS) or cleaved amplified polymorphic sequence (CAPS) markers is low due to the limited size of the fragments. A more efficient approach to map genic DNA polymorphisms in pseudo test cross mapping populations, referred to as "blind" mapping, has recently been reported (Studer *et al.* 2009). "Blind" mapping based on HRM allows for the separation of genotypes segregating in a mapping population, and thereby determining allele segregation for linkage mapping, even though type and number of allelic DNA polymorphisms in an amplified fragment are unknown. Thus, this technology makes it possible to screen and genotype DNA polymorphisms in ryegrass genes orthologous to identified timothy DEFs in a single step.

This study aimed at *1*) determining the relative water stress tolerance of *P. pratense, P. bertolonii* and *P. phleoides* species, *2*) identifying and annotating genes involved in water stress response using cDNA-AFLP and *3*) mapping orthologous genes putatively involved in water stress response in perennial ryegrass.

Materials and methods

Three randomly selected genotypes of *Phleum pratense* L. (cv. Žolis)*, Phleum bertolonii* DC (wild ecotype 2518) and *Phleum phleoides* (L.) H. Karst. (wild ecotype 2718) were grown in polyethylene pots (1 dm^3) with peat substrate and under regular irrigation and fertilization in a greenhouse for two months. When the plants reached tillering stage, they were moved into a walk-in growth chamber *PE 2412 UY-LX* (*Angelantoni Industrie*, Massa Martana, Italy) with 16-h photoperiod, irradiance 31.5 μmol m^{-2} s^{-1}, temperature of 20 ± 2 °C and relative

humidity of 60 ± 10 %. One group of each genotype was deprived of water while the second group was watered daily and used as a control. During the water stress treatment, three fully expanded leaves of each of the species were detached on day 1, 3, 5 and 6 (2 h after dark period) for leaf relative water content (RWC) measurement according to White *et al*. (1992).

For chlorophyll fluorescence determinations, three fully expanded leaves were measured daily after 2 h of dark adaptation with chlorophyll fluorometer *OS-30p* (*Opti-Science*, Hudson, NH, USA). This fluorometer directly measured variable to maximum fluorescence ratio (F_v/F_m) which was considered as a characteristic of quantum efficiency of photosystem 2.

Analysis of variance (*ANOVA*) (Hill and Lewicki 2007) was performed for all measured traits (total of 540 measurement points) in order to test the significance of variation among accessions. The coefficient of correlation between F_v/F_m and RWC was based on the whole data set.

Leaf samples from stressed and control plants were collected on the sixth day of the experiment, immediately frozen in liquid nitrogen and stored at -80 °C for RNA isolation with the plant *RNeasy* mini kit (*Qiagen*, Valencia, CA, USA). Quality and quantity of the RNA was checked by running 5 mm^3 of the total RNA on a 1 % agarose gel. Poly(A)$^+$RNA was isolated from the total RNA by using the *Dynabeads* mRNA purification kit (*Invitrogen*, Carlsbad, CA, USA). Poly(A)$^+$RNA was reverse transcribed with an anchored oligo-dT_{11} primer using a *RevertAid*^{TM} *H Minus* first strand cDNA synthesis kit (*Fermentas*, Vilnius, Lithuania) according to the manufacturer's protocol. Reverse transcription reaction product (0.02 cm^3) was used in a second strand cDNA synthesis reaction by adding 30 U of DNA *Polymerase I* (*Fermentas*). The double-stranded cDNA was purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and quantified by running 8 mm^3 of cDNA on 1 % agarose gel.

The cDNA-AFLP procedure was conducted as described by Bachem *et al.* (1998) with minor modifications. cDNA (50 ng) was digested with 10 U *Taq*I at 65 °C for 10 min, and then immediately digested with 10 U of *Ase*I at 35 °C for 35 min. The two steps of digestion were conducted in a *Fast Digest* buffer (*Fermentas*) in a total volume of 0.05 cm^3. 50 pM *Ase*I and 5 pM *Taq*I adapters were ligated to digested cDNA using 1 U of T4 ligase supplemented with *Taq*I buffer (*Fermentas*). PCR reaction solution (0.05 cm^3) for pre-amplification contained 1 mm^3 ligation mix, 2 µM of each primer with one selective nucleotide, 0.25 mM dNTP mix, 2 mM $MgCl_2$, and 1.25 U *Taq* polymerase (*Fermentas*). The PCR reaction was conducted on the *Mastercycler* gradient thermo cycler (*Eppendorf*, Hamburg, Germany) at: 94 °C, 1 min; 25 cycles of 94 °C, 30 s; 55 °C, 30 s; and 72 °C, 1 min; then followed by 10 min at 72 °C for a final extension. For selective amplification, the PCR solution included 10 mm^3 of 20× diluted pre-amplification product, 0.25 µM of *Ase*I selective primer with three selective nucleotides, 1.5 µM of *Taq*I selective primer with two selective nucleotides, 2 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.4 U *Taq* polymerase in 20 mm^3 total reaction volume. The PCR reaction was performed using the following program: 12 cycles: 94 °C, 30 s; 65 °C (-0.7 °C/cycle), 30 s; 72 °C, 1 min and 24 cycles: 94 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min; followed by a final extension step of 10 min at 72 °C. The selective PCR products were loaded onto a 2 % agarose gel for fractionation by electrophoresis at 100 W for 2.5 h. The fractionated fragments on the gel were then visualized by using ethidium bromide and UV radiation.

For each primer combination, the final PCR products of water deficit-stressed and control plants were loaded next to each other on the gel for comparison. The reproducibility of differentially expressed fragments (DEFs) was tested by repeating the PCR amplifications 2 to 3 times with each of the primers analyzed. Only robust and repeatable bands were included in this study. DEFs were excised from the agarose gel with a sterile surgical blade, purified from agarose with a *QIAquick* gel extraction kit (*Qiagen*) and eluted with 0.03 cm^3 of sterile water. DNA fragments were cloned into the *pCR*®*-Blunt* vector using the *Zero Blunt*® PCR cloning kit (*Invitrogen*) according to manufacturer's instructions. Plasmid DNA was isolated using the *GenElute*^{TM} *Plasmid Miniprep* kit (*Sigma-Aldrich*, St. Louis, MO, USA) and sequenced by *EuroFins MWG Operon* (Ebersberg, Germany).

DNA sequences of DEFs were clustered and assembled using the software *SEQMAN* (*DNAStar*, Madison, WI, USA). The sequences of established contigs were blasted against the non-redundant (nr) protein database of *Genbank* with *BLASTX*. PCR primers were designed using *Primer3* software (Rozen and Skaletsky 2000). The primer pairs were designed to amplify fragment lengths of approximately 200 bp and to fulfil the following criteria: *1*) primer length of 18 to 24 bp (target: 20 bp), *2*) Tm of 59 to 61 ºC (target: 60 ºC; $\Delta Tm < 1$ ºC) and *3*) GC content of 40 to 70 %.

For each primer pair, the annealing temperature to amplify a highly specific PCR product was determined using gradient PCR ranging from 58 to 72 ºC and VrnA parental DNA as template. PCR amplification in the VrnA population along with the parents (NV#20F1-30 and NV#20F1-39) and the grandparents (Falster and Veyo) was performed in a total volume of 10 mm^3 containing *1X LightScanner* master mix (*Idaho Technology*, Salt Lake City, UT, USA), 0.10 µM of each forward and reverse primer, and 20 ng of DNA. In order to avoid evaporation of the PCR mix during PCR amplification and HRM analysis, reactions were covered with 14 mm^3 mineral oil. The PCR amplification was conducted in a *PTC-225* Peltier thermo cycler (*MJ Research*, Waltham, MA, USA) as follows: initial denaturation of 2 min at 95 ºC, followed by 40 cycles of 30 s at 94 ºC, 30 s at the optimal annealing temperature for each primer pair and a 30 s elongation step at 72 ºC. For heteroduplex formation, a final cycle of 30 s at 94 ºC

and 30 s at 25 ºC was added. HRM analysis was performed using the *LightScanner* instrument (96-well plate format) and the *LightScanner*® and Call-IT® software modules (*Idaho Technology*). The HRM analysis was carried out following PCR at temperatures from 80 to 95 ºC at steps of 0.05 ºC, each step with a 1 s hold.

In order to map DEFs in perennial ryegrass, a subset of 92 F2 genotypes along with the parents (NV#20F1-30 and NV#20F1-39) and the grandparents (Falster and Veyo) of the VrnA perennial ryegrass mapping population was used. Map construction was carried out according to Jensen *et al.* (2005) using the Haldane mapping function of the software *JoinMap 4* (Van Ooijen 2006).

Table 1. HRM primer sequences along with PCR results and the map position given in cM in the VrnA perennial ryegrass mapping population

Primer name	HRM primer sequence forward (5' - 3')	HRM primer sequence reverse (5' - 3')	HRM results	Map position [cM]
Ti_Con_094	AAGAGCAAGAAGTCGGTGGA	TCTCTTTGCATGGTTTGCTG	polymorphic	LG 4 39.8
Ti_Con_095	TAGAGGTTTGGCCACGCATA	CACAATGCTCACCGAGTTGT	unspecific	not mapped
Ti_Con_096	CTTACCTGCCACCGCTCTCG	TGCTTCAGGAGGGCCTCGG	monomorphic	not mapped
Ti_Con_097	TGGGCTGAAGAGAACGAAGT	GAGGAGTCTAAGGCCCTCGT	no amplification	not mapped
Ti_Con_098	AGCTACCTTGGCTCCCAGA	GCAGCCACAAAACCTTCAGT	no amplification	not mapped
Ti_Con_099	GACGCACTTGAAGCATACGA	CTATTAGGCTCCCCGCTCTT	polymorphic	LG 5 50.9
Ti_Con_100	TGGTTTTGTTGTGAAACAGTCT	GCACACGATCCAACTCACAC	no amplification	not mapped
Ti_Con_102	CCATGAGAATTTTCCGCATT	TGCAAGCAAAACCACTTGAA	polymorphic	LG 6 11.5
Ti_Con_103	GGAAATGCCAATGGATGAGT	AAGGCCCTCCAAATTCAGAT	polymorphic	LG 3 24.6
Ti_Con_104	ATTCATGCCTTAAGCCCGGA	CAACTTCTTGCAGCCAGAACC	polymorphic	LG 2 104.6
Ti_Con_105	CATTCACGGATTGCATTGAG	TGATAGCGGAAAGAGGCATT	polymorphic	LG 4 39.2
Ti_Con_106	CCATGTGCGTTACTTGGTTG	GGGTGGGACTTGAAACTGAA	no amplification	not mapped
Ti_Con_107	CTTGGAGGTGCCTTTGGTAA	GGAACAGCTTGTTGCTCTCC	no amplification	not mapped
Ti_Con_108	CATCCTAGCCCAACAAAGGA	TCTGCATATCTGCGAACAGG	polymorphic	LG 4 95.2
Ti_Con_109	CGTGAACGGATTATCTTCATTG	TCCTCCTGGACCATTGATGT	polymorphic	LG 7 17.7
Ti_Con_110	CCAGTTGACTGGTTCTTTACC	CTTGGTTTTGTTCAGGTTAGC	monomorphic	not mapped
Ti_Con_111	AGTCCTGACCGAATCAACTCA	TGCCACAGATAGCTGAATCC	no amplification	not mapped
Ti_Con_112	CACATCACTGGCGTCATTCT	ATTGCATTTTTGGCCAATGT	no amplification	not mapped
Ti_Con_113	CTCCTGGCAAAGGCAAAAGC	GCAACAAAAGGGAGACACCCTT	polymorphic	LG 1 33.9
Ti_Con_114	GTATTGTGTGCAGAAGTGGAGG	CAAGGAAAACCAAGACTCAAAAAAT	unspecific	not mapped
Ti_Con_115	AGCGGTCTCCTCAAGAACAA	CGCCCACTCTCTACAAGGAA	monomorphic	not mapped
Ti_Con_116	GAGAAGATGGCGTTGAAGGAA	CAGCTGCAACGGCACCA	monomorphic	not mapped
Ti_Con_117	CTGGGTACCCTCCATTCTCC	TGATGCTGCCCTTGGAACTG	polymorphic	LG 2 101.6
Ti_Con_118	AGTGCTGGACTGCCACAC	TTGACGGCGATGTGGGA	unspecific	not mapped
Ti_Con_119	GGAGGAGTTTGGAAAGATGAA	AATTTCGTCGATACCCTTCTC	monomorphic	not mapped
Ti_Con_121	GCTGAATGCTGGATGGCTC	CTATATGGCTTATTACAAATTCACAT	polymorphic	LG 3 24.6
Ti_Con_122	GAGGATGGCTGGAAGTTCA	AAGAATTGGCCATGTCGTACT	no amplification	not mapped
Ti_Con_124	CTCAGGCTGGATACACCACA	ACTCAAGGCAAGCTGCGTAT	no amplification	not mapped
Ti_Con_125	GGTTCAGAGATGGTTCTTCATTT	AGCCACAAATGAAGAGAAGCC	polymorphic	LG 1 26.8

Results

Plant response to water stress was evaluated during 6 d after cessation of watering. RWC and F_v/F_m in all *Phleum* species were unaffected for the first 2 d of the experiment. Plants began to discolour and wilt at the third day after water deprivation and RWC of *P. pratense* and *P. bertolonii* decreased by 34 and 32 %, respectively, while that of *P. phleoides* decreased by only 17 % after 6 d of drought stress. Final RWC values declined to 53 % for *P. pratense*, 58 % for *P. bertolonii* and 73 % for *P. phleoides* (Fig. 1).

The F_v/F_m ratio decreased from 0.717 to 0.688 for *P. pratense* and from 0.711 to 0.619 for *P. bertolonii* during the first 3 d of water stress, while *P. phleoides* maintained the same F_v/F_m ratio at a value of 0.726 (Fig. 2). Final F_v/F_m ratio reached 0.490 for *P. pratense* and 0.457 for *P. bertolonii*. For both traits, no significant differences between *P. pratense* and *P. bertolonii* were detected, whereas unstressed and stressed plants differed significantly ($P < 0.001$). In contrast, no significant differences between unstressed and stressed *P. phleoides* plants were found and *P. phleoides* differed significantly ($P < 0.01$) from both *P. pratense* and *P. bertolonii*. The correlation between F_v/F_m and RWC was high ($r = 0.95$).

cDNA-AFLP analysis was performed using 23 primer pair combinations on stressed and unstressed plants and revealed 189 transcript derived fragments (TDFs) with an average of 8.2 fragments per primer pair. The size of these fragments ranged from 100 to 1 600 bp. Of these

Table 2. Functional annotation of the 19 contigs revealing significant (E <-10) sequence similarities in *BLASTX* search against the non-redundant (nr) protein database of *Genbank* along with the contig size and accession numbers in *GenBank EST* database.

Contig number	Status	Size [bp]	Top BLASTX hit to GenBank	*E* value	Homologue protein description	Protein function	Plant
Ti_Con_094	down-regulated	709	gi|226500714|	9.0E-94	nitric oxide synthase-interacting protein homolog	mediating in plant defense responses	*Z. mays*
Ti_Con_095	down-regulated	1064	gi|195654965|	4.0E-104	UDP-sugar pyrophospharylase	distribution of glycosyl units to starch, sucrose and cell-wall polysaccharides	*Z. mays*
Ti_Con_096	down-regulated	537	gi|11990897|	2.0E-43	ribulose-1,5-bisphosphate carboxylase	carboxylation or the oxygenation of ribulose-1,5-bisphosphate	*T. aestivum*
Ti_Con_103	down-regulated	878	gi|110224485|	4.0E-43	transcription factor MYBS3	drought tolerance	*Z. mays*
Ti_Con_104	down-regulated	995	gi|5478530|	8.0E-101	UVB-resistance protein UVR8	acting in UV-B signal transduction pathway	*A. thaliana*
Ti_Con_105	down-regulated	687	gi|56160523|	1.0E-94	RelA/SpoT homologs (RSH1)	abiotic stresses-responsive proteins	*O. sativa*
Ti_Con_107	down-regulated	1121	gi|56160523|	6.0E-68	transcriptional corepressor SEUSS	encoding of a regulator of floral homeotic gene *AGAMOUS*	*A. thaliana*
Ti_Con_108	down-regulated	751	gi|18398450|	7.0E-108	RNA binding/ribonuclease	RNA binding	*A. thaliana*
Ti_Con_109	down-regulated	458	gi|195612324|	6.0E-75	ATP-dependent Clp protease proteolytic subunit 2	abiotic stresses-responsive protein	*Z. mays*
Ti_Con_110	down-regulated	699	gi|224589376|	2.0E-83	leucine-rich repeat receptor-like protein kinase	phosphorylation of proteins within the cell, resulting in transduction of the signals	*A. thaliana*
Ti_Con_113	down-regulated	705	gi|129915|	3.0E-90	phosphoglycerate kinase	transference of phosphate group from 1,3-biphosphoglycerate to ADP, forming ATP and 3-phospho-glycerate	*T. aestivum*
Ti_Con_114	down-regulated	836	gi|15232845|	3.0E-92	mitochondrial-processing peptidase beta subunit	responsibility for the proteolytic processes	*A. thaliana*
Ti_Con_115	down-regulated	960	gi|75165389|	8.0E-161	sucrose-phosphatase (EC 3.1.3.24)	major role in sucrose biosynthesis	*O. sativa*
Ti_Con_116	down-regulated	957	gi|75253362|	1.0E-53	fact complex subunit ssrp1-b	involvement in mRNA elongation, DNA replication and DNA repair	*O. sativa*
Ti_Con_117	down-regulated	991	gi|75144954|	3.0e-107	protein phosphatase 2C containing protein	negative regulators of ABA signalling	*O. sativa*
Ti_Con_118	up-regulated	886	gi|158142210|	4.0e-159	elongation factor 1-alpha	catalysation of binding of aminoacyl-transfer RNAs to the ribosome	*L. perenne*
Ti_Con_119	down-regulated	1077	gi|20302471|	6.0E-22	ferredoxin-NADP(H) oxidoreductase	linear photosynthetic electron transport	*T. aestivum*
Ti_Con_122	up-regulated	349	gi|128189|	5.0E-31	nitrate reductase [NADH]	catalyzation of the last three steps in the reduction of nitrate to NH_3	*H. vulgare*
Ti_Con_125	down-regulated	463	gi|17887465|	7.0E-76	phosphoethanolamine methyltransferase	providing the precursor phosphocholine in glycero-phospholipid metabolism	*T. aestivum*

TDFs, 89 (47.1 %) were identified as being differentially expressed (presence/absence) after 6 d of water deprivation. The expression pattern included 22 (24.8 %) up-regulated and 67 (75.2 %) down-regulated DEFs. 64 fragments were isolated from agarose gels, of which 52 were successfully cloned and sequenced.

The forward and reverse sequences of 52 DEFs were assembled into 29 contigs and used to design primer pairs for linkage mapping in the VrnA ryegrass mapping population. These contigs were deposited to the *GenBank EST* database with accession numbers of GW883523 to GW883554. A total of 19 contigs revealed significant (E value < $1e^{-10}$) sequence similarities in a *BLASTX* search against the nr protein database of *Genbank* (Table 2). HRM analysis was performed on 92 F_2 individuals from the VrnA mapping population along with the parents and grandparents. Out of a total of 29 primer pairs tested (Table 1), five amplified monomorphic fragments,

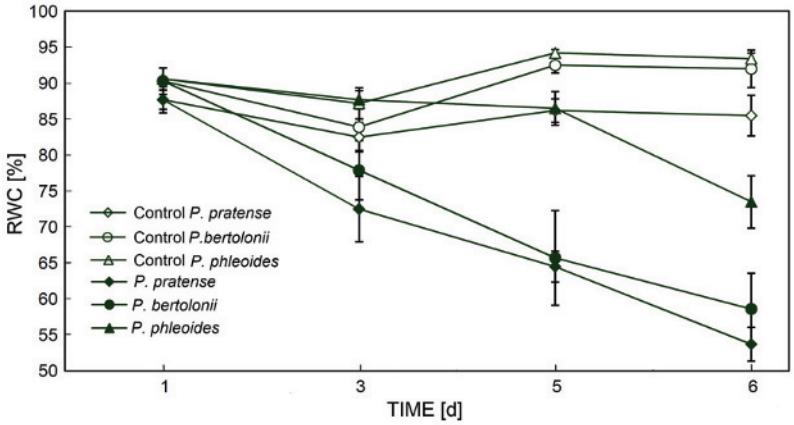

Fig. 1. Relative water content (RWC) in flag leaves of *P. pratense*, *P. bertolonii* and *P. phleoides* plants under water-stress treatment, compared with those of control plants. Means ± SD, $n = 9$.

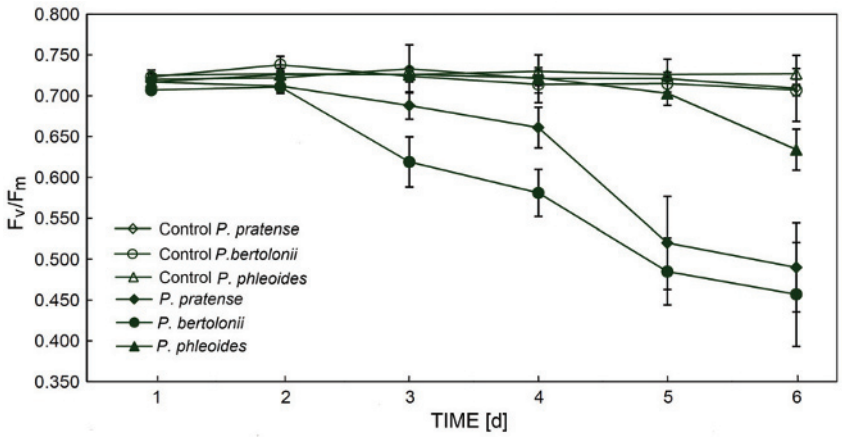

Fig. 2. Maximum potential quantum efficiency of photosystem 2 (F_v/F_m) in flag leaves of *P. pratense*, *P. bertolonii* and *P. phleoides* plants under water-stress treatment, compared with those of control plants. Means ± SD, $n = 9$.

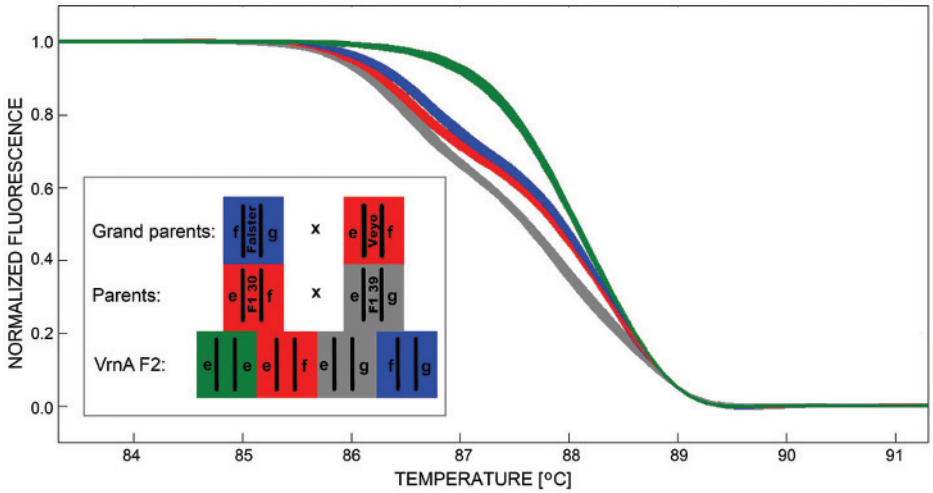

Fig. 3. HRM profiles of Ti_Con_094 based on 92 F_2 genotypes along with the parents and the grandparents of the VrnA perennial ryegrass mapping population. Heterozygous genotypes ef (red) and eg (grey) were each represented in the parents and in one grandparent as well as in 23 and 27 F_2 individuals, respectively. The homozygous genotype ee (green, represented by 19 F_2 individuals) can be clearly separated from the heterozygous genotypes. Genotype fg (blue) is represented by 23 F2 individuals. For genetic linkage mapping, Ti_Con_094 segregated in a 1:1:1:1 ratio in the progeny and followed the segregation type efxeg (Maliepaard *et al.* 1997).

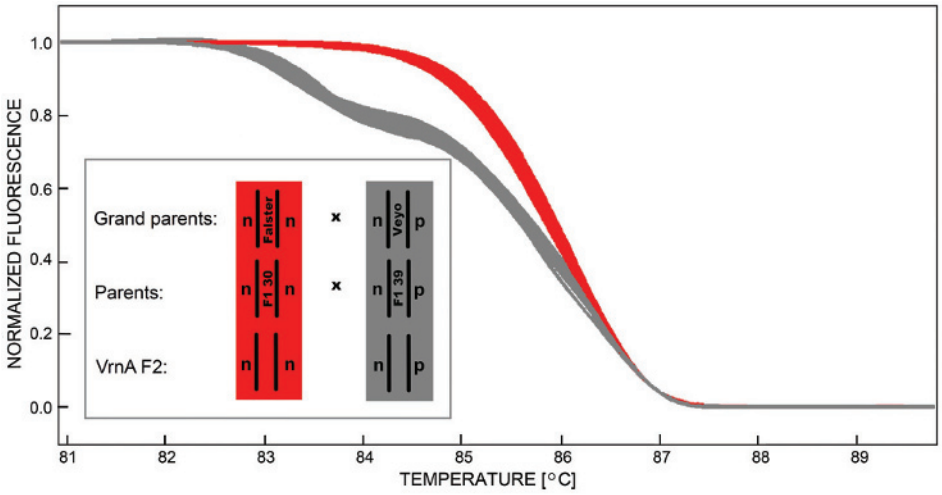

Fig. 4. HRM profiles of Ti_Con_099 based on 92 F_2 genotypes along with the parents and the grandparents of the VrnA perennial ryegrass mapping population. Ti_Con_099 is segregating in a 1:1 ratio in the F_2 progeny. Both homozygous (red) and heterozygous (grey) genotypes are represented in one grandparent, in one parent and in 52 and 40 F_2 mapping individuals, respectively. For genetic linkage mapping, Ti_Con_099 followed the segregation type nnxnp (Maliepaard *et al.* 1997).

indicating that no DNA polymorphism was present in the amplified PCR fragment of VrnA. Further 9 DEFs could not be amplified in the VrnA mapping population. Three primer pairs amplified unspecific or more than one PCR product. The remaining 12 DEFs (Ti_Con_094, Ti_Con_099, Ti_Con_102, Ti_Con_103, Ti_Con_104, Ti_Con_105, Ti_Con_108, Ti_Con_109, Ti_Con_113, Ti_Con_117, Ti_Con_121 and Ti_Con_125) were genotyped by HRM in the VrnA mapping population and revealed from 2 to 4 different melting curve shapes. According to the melting curve shapes, the allelic segregation patterns of DEFs were recovered and used for linkage mapping (Figs. 3 and 4).

Twelve DEFs putatively involved in water stress response in timothy were mapped on the VrnA linkage map containing a total of 222 markers, between 24 and 37 on each linkage group (LG). The 222 markers (136 EST-SSR, 24 genomic SSR, 23 AFLP, 19 CAPS and 20 HRM markers) were selected to be evenly distributed along the LGs and to span the entire genome. The DEFs were located on seven LGs: three on LG 4, two on LG 1, LG 2 and LG 3 and one each on LG 5, 6 and 7 (Fig. 5, Table 1). The total map length was 736 cM, ranging from 88 cM of LG 1 to 166 cM of LG 2.

Discussion

Plant adaptation to environmental stresses is regulated through multiple physiological mechanisms at the cellular, tissue, and whole-plant level (Hazen *et al.* 2005, Yamaguchi-Shinozaki and Shinozaki 2005, Ito *et al.* 2006). Identification and detailed analysis of candidate genes involved in water stress may enable us to elucidate the molecular basis for such complex responses.

The investigated period of water stress treatment covered early dynamic changes of plants responding to the stress on physiological as well as molecular level. The experiment clearly demonstrated the existence of different water stress response in *Phleum* species. *P. phleoides* plants maintained higher leaf RWC and showed less reduction in F_v/F_m under water stress as compared to *P. pratense* and *P. bertolonii* plants. These results suggest that *P. phleoides* may have evolved under conditions of limited water availability.

RWC has been used as a direct indicator for water stress (Flower and Ludlow 1986). Wang and Bughrara (2007) used the RWC for measuring water stress in *Festuca mairei*. These authors also used RWC for evaluation of drought tolerance for *Festuca mairei*, *Lolium perenne* and their progeny (Wang and Bughrara 2008). *In vivo* F_v/F_m is considered as good indicator for response of plant photosynthesis to water stress. Recent improvements in measurement techniques have made this method an important tool in stress physiology and environmental research (Roháček 2002, Urban 2008).

Several of the drought up- and down-regulated genes identified by cDNA-AFLP had previously been reported as being differentially expressed upon drought stress in *Fesuca mairei* (Wang and Bughrara 2007), underlining the power of cDNA-AFLP for gene discovery in plant species for which no large scale sequence information is available (Bachem *et al.* 1996). In most of the previous studies, a large number of differentially expressed genes were up-regulated upon water stress. However, Wang and Bughrara (2007) have shown that down-regulation may also play important roles in water stress response. Gene expression patterns of *Festuca mairei* under drought included mostly down-regulated DEFs (54.3 %), while up-regulation accounted for only 30 % of all DEFs. The

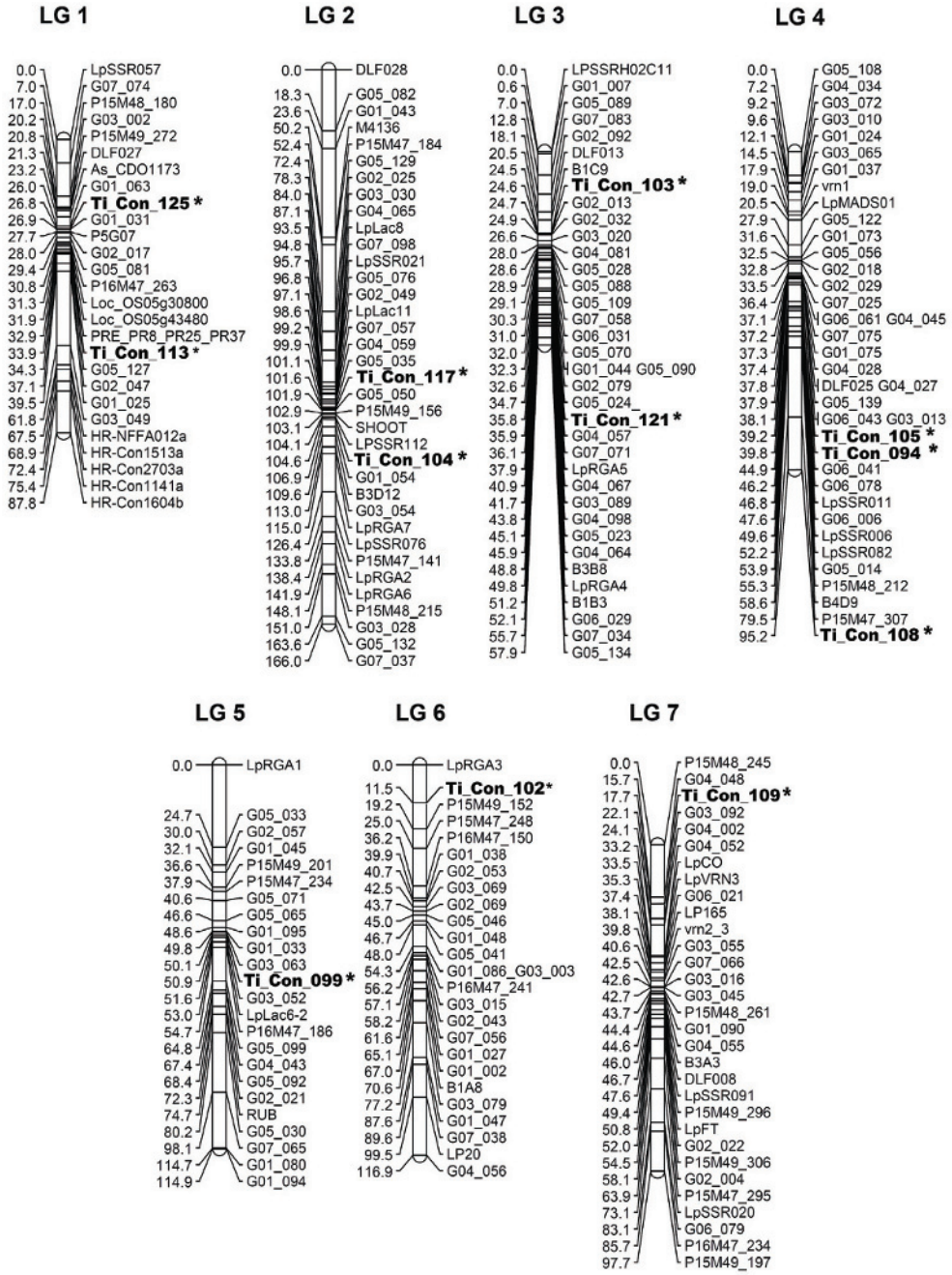

Fig. 5. Genetic linkage map of the perennial ryegrass VrnA population based on 184 F2 individuals and 222 genetic markers. Putative water stress response genes mapped in this study are indicated in *bold* with an *asterisk*. Scale units are given in cM.

remaining 16.7 % were attributed to transient changes in expression. We observed even higher proportions of down-regulated DEFs (75.2 %). However, our experiment was designed to compare gene expression patterns of plants after 6 d of water deprivation with those of control plants. Therefore we did not detect any dynamic changes of gene expression, such as transient and up-then-down expression, which would account for down-regulation after 6 d of water deprivation. This might explain why some of the genes detected as being down-regulated in our study were found to be up-regulated in other studies, as it was the case for Ti_Con_103.

Nineteen genes putatively involved in water stress response showed homology to known proteins described in other *Poacea* species such as rice, wheat and maize as well as the plant model species *Arabidopsis thaliana*. These genes can be classified into two groups: those that produce direct protectors against environmental stresses and those that regulate gene expression and signal transduction in the stress response. The products of the genes in the first group have the ability to function in stress tolerance, such as enzymes involved in sugar

metabolism and biosynthesis. We have isolated sucrose-phosphatase, UDP-glucose pyrophosphorylase and ribulose-1,5-bisphosphate carboxylase DEFs. Under water stress, sugars probably function as osmolytes in protecting cells from dehydration (Cushman and Bohnert 2000). The second group contains regulatory proteins involved in regulation of signal transduction and gene expression in stress responses. Transcription factor MYB S3 was recently shown to play a critical role in cold adaptation in rice and activate several genes involved in various stress response and/or tolerance (Su *et al.* 2010). Over-expression of nitric oxide synthase-interacting protein (NOSIP) induces translocation of NOS from the plasma membrane to intracellular compartments, thereby impairing NO production, which has been described as a physiological mediator in plant defence responses (Crawford 2006). The functional characterization of RelA/SpoT homologs (RSH1) is consistent with a role for guanosine pentaphosphate [(p)ppGpp] in mediating a stress-induced defence system in plants (Misuzava *et al.* 2008). Protein phosphatase type 2C (MP2C) was found to function as a negative regulator of the stress-activated MAPK pathway that is activated by cold, drought, touch, and wounding (Meskiene *et al.* 1998). To date, the functions of the remaining genes are not fully understood. Among ten water stress responsive genes mapped in the VrnA population, three of them (Ti_con_102, Ti_Con_121 and Ti_Con_099) showed no homology to known proteins of other species. These findings suggest the identification of novel genes involved in mediation of the response to water deficit and need further validation. The results of this study provide clues for the development of functional markers for water stress response in timothy.

HRM is a closed-tube post-PCR method that can be applied for "blind" mapping and has advantages of speed, simplicity and low cost (Studer *et al.* 2009). However, the main benefit of "blind" mapping is that DEFs can be mapped without allelic sequence information generated by time and cost intensive amplicon cloning and sequencing. In this study, "blind" mapping by HRM was successfully used to map water stress response genes of timothy in the perennial ryegrass F2 mapping population VrnA. In total, 12 putative water stress response genes were mapped on seven perennial ryegrass linkage groups. The candidate genes Ti_Con_117 and Ti_Con_104 were mapped in the region of LG 2, where QTL for wilting in the WSC F_2 mapping family under drought treatment were found (Turner *et al.* 2008). The candidate genes Ti_Con_113 and Ti_Con_099 were mapped on LG 1 and 5, respectively, in close proximity to the QTL for drought resistance, such as plant survival, rehydration, tiller survival and regrowth after rewatering (Turner *et al.* 2008). The QTL for drought resistance on chromosome 3 of fescue has been exploited in crop improvement programmes of ryegrass through their independent introgression into homoeologous regions of the ryegrass genome (Humphreys *et al.* 2005). The detailed study by Alm *et al.* (2011) demonstrated the importance of meadow fescue chromosomes 1, 3, 4, 5 and 6 where the QTLs for growth under moderate (about 2 months without water) and severe (about 5 months without water) drought were located. A QTL for long-term drought tolerance on fescue chromosome 3 (homologeous to ryegrass chromosome 3) support evidence that this region is an excellent source of tolerance towards drought stress (Alm *et al.* 2011). Aligning the genetic map to rice, where detailed genomic information has been accumulated, could provide us more information about the QTL or genes of interest (Devos 2005). The candidate gene Ti_Con_099 is located in the same chromosomal region as QTLs tagged for root morphology and leaf rolling in rice under drought conditions (Champoux *et al.* 1995), while Ti_Con_097, Ti_Con_108, Ti_Con_109 and Ti_Con_125 co-localized with QTLs for root and shoot morphology in two contrasting moisture regimes (Hemamalini *et al.* 2000).

In conclusion, mapping of water stress response genes from timothy in perennial ryegrass provides further support for the concept of using diploid perennial ryegrass as a model for genetically more complex grassland species, such as timothy. The localization of these genes on the ryegrass map will facilitate further characterization of water response in timothy. Moreover, the genomic data obtained in ryegrass could be further used to validate the described water stress response genes by linkage or association analysis, thus providing a first step towards grassland cultivars with improved tolerance to water deficit.

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