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ORIGINAL PAPER

Polymorphism of a new Ty1-*copia* retrotransposon in durum wheat under salt and light stresses

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Abstract Long terminal repeat retrotransposons are the most abundant mobile elements in the plant genome and play an important role in the genome reorganization induced by environmental challenges. Their success depends on the ability of their promoters to respond to different signaling pathways that regulate plant adaptation to biotic and abiotic stresses. We have isolated a new Ty1-*copia*-like retrotransposon, named *Ttd1a* from the *Triticum durum* L. genome. To get insight into stress activation pathways in *Ttd1a*, we investigated the effect of salt and light stresses by RT-PCR and S-SAP profiling. We screened for *Ttd1a* insertion polymorphisms in plants grown to stress and showed that one new insertion was located near the resistance gene. Our analysis showed that the activation and mobilization of *Ttd1a* was controlled by salt and light stresses, which strengthened the hypothesis that stress mobilization of this element might play a role in the defense response to environmental stresses.

Introduction

Plant growth and productivity is under constant threat from environmental challenges in the form of various abiotic and biotic stress factors. Plants are frequently exposed to a plethora of abiotic conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metals. Soil salinity and high light intensity have a significant impact on agricultural production, reducing crop quality and productivity, especially when these stresses occur during the early phases of seedling growth. Salt tolerance is a complex trait involving responses to cellular osmotic and ionic stresses and their consequent secondary stresses and entire plant coordination (Wang et al. 2003). The complexity and polygenic nature of salt tolerance are important factors contributing to the difficulties in breeding salt-tolerant crop varieties (Zhu 2000). Light also has a pronounced effect on gene expression, particularly during the early photomorphogenetic development of plants (Soitamo et al. 2008). Indeed, besides its function via photoreceptors, light exerts effects on gene expression via the photosynthetic apparatus, the function of which can be strongly modulated by various environmental stress conditions.

Recent evidence suggests the possible generation of genetic plasticity in response to stress through the mobilization of retrotransposons, the most widespread eukaryotic mobile elements (Grandbastien et al. 2005). Stress and external challenges, including microbial attacks, are known to be major factors activating retrotransposons expression in a wide range of organisms such as yeast (Bradshaw and McEntee 1989), drosophila (Strand and McDonald 1985), mammals (Liu et al. 1995) and plants (Grandbastien 2004, 2005; Salazar et al. 2007). Retrotransposons have contributed significantly to the remarkable variations in genome size and may also have contributed to genomic evolution by changing the structures and expression patterns of genes (White et al. 1994).

The transposable elements (TEs) are generally classified by first following their transposition intermediates and then

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their structure and sequence homologies. Retrotransposons belonging to class I can be distinguished as long terminal repeats (LTR) and non-LTR retrotransposons. LTR retrotransposons are further divided by the order of the genes for reverse transcriptase (RT), RNaseH and integrase, and by sequence similarity into the Ty1-*copia* and *Ty*3- *gypsy* group. Class I element moves via an RNA intermediate that is reverse transcribed into extrachromosomal DNA and inserted into the genome. This process can reach such an extent that such elements are now considered to be the primary factor in genome size variation in plants, other than polyploidization (Piegu et al. 2006).

Most of the TEs found in the complex eukaryotic genome are not functional, either because they are the target of silencing processes that impede their proliferation in the genome (Slotkin and Martienssen 2007) or because they have been structurally altered by mutations (Vitte et al. 2007). The "inactive" elements cannot contribute to genome evolution through retrotransposition (Fantaccione et al. 2007). Despite their propensity to invade and densely populate plant genomes, only a few transpositionally active TEs have been identified so far in plants. Evidence for element mobility is available for the maize *Bs*1 and *Zeon*-1 elements (Hu et al. 1995), the tobacco *Tnt1* and *Tto1* elements (Grandbastien et al. 1997; Hirochika et al. 1996a), the *Tos* and *Lullaby* elements of rice (Hirochika et al. 1996a, b; Picault et al. 2009), and the *BARE*-1 of barley retrotransposon (Suoniemi et al. 1996). The expression of the tobacco *Tnt1* and *Tto1* retrotransposons is greatly increased by several abiotic stresses including cell culture, $CuCl_2$ and salt stress (Grandbastien et al. 1997). Transposons can therefore be powerful contributors to plant gene and genome structure, and evolution (Gbadegesin et al. 2008, Kumar and Bennetzen 2000). In fact, many mechanisms are responsible for the molecular evolution of plant disease resistance genes, some of which are believed to involve TEs (Hayashi and Yoshida 2009).

A feature of some active plant retrotransposons is that they all contain *cis*-regulatory elements in their promoter (U3 region of 5′ LTR) associated with signal transduction pathways related to plant defense responses (Grandbastien et al. 1997; Pouteau et al. 1994; Takeda et al. 1999). This suggests that active plant retrotransposons are switched on by stress-associated responses because their promoter elements are similar to those of plant defense genes and may bind similar defense-induced transcription factors. In this regard, it has also been proposed that retrotransposons could have captured plant defense promoters from endogenous genes or, inversely, they could have provided their inducible promoters to some defense genes (Grandbastien 2004). If the latter hypothesis is right, the promoters of active retrotransposons will be important in studying regulatory mechanisms for plant defense.

In this study, we have identified a new Ty1-*copia*-like retrotransposons in the *Triticum durum* genome. To get some insight into the interaction between the stress activation pathways of the retrotransposons and stress-inducible genes, we investigated the expression and insertional polymorphism of one retroelement, named *Ttd1a* (*Transposon Triticum durum 1 active*), from the leaves of *T. durum* seedlings subjected to salt or light stress treatments. The role that this element might play in generating host genomic plasticity in response to environmental stresses is discussed here.

Materials and methods

Plant material

Durum wheat (*Triticum durum* Desf. Cultivar Ofanto genome constitution AABB cod.39) seeds, selected by the experimental institute for cereal research CRA (Foggia, Italy), were germinated in the dark at 21°C on filter paper moistened with deionized water. After 3 days, when the primary root was emerging from the seeds, individual seedlings were transferred to nine 2-L pots with perforated plastic tops (30 plants per pot) containing aerated distilled water. After 3 days of hydroponic culture, the water was replaced with Hoagland medium containing 10 mM KNO_3 as nitrogen source under controlled conditions (16-h photoperiod, 300 µmol m^{-2} s^{-1} PAR, thermoperiod 25/20°C day/night, 65% relative humidity). The nutrient solution was replaced every 3 days. Starting from day 10 of culture, three pots were subjected to salt stress by supplementing the hydroponic medium with 50 mM of NaCl, increased to 100 mM NaCl on day 11. The NaCl concentration was increased gradually in this way to reduce salt shock to plants. The control plants in the other pots were grown without adding NaCl. Starting from day 15 of culture, three pots of control plants were subjected to high light (900 µmol m^{-2} s^{-1} PAR, UV light was not present in our artificial light). The control plants and salt-stressed ones in the other pots were grown in 300 µmol m^{-2} s^{-1} PAR. After 20 days of hydroponic culture (10 days of salt treatment or 5 days of high light treatment), 4 h after the beginning of the light period, leaves were harvested and used immediately for assays.

Nucleic acid extraction

Total DNA and RNA were isolated from three different biological samples, each one constituting a pool of 30 plants. DNA was extracted from the fresh leaf tissues using the CTAB procedure of Doyle and Doyle (1987) followed by treatment with RNase A. Total RNA was prepared from leaf tissue using the RNeasy plant RNA extraction kit (Qiagen) and poly(A)$^+$. RNA was purified from ~700 µg of

leaf RNA using an Oligotex mRNA purification kit (Qiagen). First-strand cDNA was obtained using the Superscript cDNA synthesis kit (Invitrogen).

Primers

The following oligonucleotides were used as primers for degenerated oligonucleotide primers-PCR (DOP-PCR), RT-PCR, isolation RNaseH-LTR regions and S-SAP assay: RT1 (5′-GATGTDAARACKRCNTTYYTD-3′), RNaseH1 (5′-CCTCACATCWATRTGYTTBGW-3′); T.d.1 (5′-GAGTTTGCTCTCAAGGACTTGG-3′), T.d.2 (5′-AACCTTGCTAAGGTTGAGAT-3′), P1 (5′-CCTGACCCTGAAGTACCCCA-3′), P2 (5′-CGTCATGCAGCTCATAGCTC-3′); RT (5′-GCATGGATTTCTGGAAGAAGA-3′), *Eco*R1 (5′-CCCAAAGCCTATCCTCGAATTC-3′), Adat.1 (5′-AATTCGAGGATAGGCTTTGGG-3′), Adat.2 (5′-CCCAAAGCCTATCCTCG-3′); PPT-LTR (5′-TAGATTGAGGGGGAGTGTTAGA-3′), *Eco*R1 + C (5′-CCCAAAGCCTATCCTCGAATTCC-3′), *Eco*R1 + T (5′-CCCAAAGCCTATCCTCGAATTCT-3′), *Eco*R1 + A (5′-CCCAAAGCCTATCCTCGAATTCA-3′), *Eco*R1 + G (5′-CCCAAAGCCTATCCTCGAATTCG-3′).; LTR-7 (5′-GTATCAGAGCCAAGAGGTCT TG-3′).

DOP-PCR amplification of RT/RNaseH motifs sequenced

Total genomic DNA (2 µg) was completely digested with *Eco*RI as specified by the manufacturer (Roche) and digested samples (100 ng) were used for subsequent PCR amplifications with degenerate oligonucleotide primers (RT1/RNase H1). The sense RT1 and the antisense RNaseH1 degenerate oligonucleotide primers were used, respectively, corresponding to the conserved RT (DVKTA(T)FL) and RNaseH (T(S)KHIDVR) peptide motifs of the Ty1-*copia* group retrotransposons (Guyot et al. 2005; Hirochika et al. 1996a, b; Theologis et al. 2000). PCR amplifications of the 1-kb fragments were performed in 20-µl reaction volume containing 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 200 µM of each oligonucleotide, and 1 U of Hot-star *Taq* DNA Polymerase (Qiagen). Cycling included a denaturation step at 95°C for 15 min, followed by 30 cycles at 94°C for 1 min, 46°C for 1 min, 72°C for 1 min and a final extension of 10 min. Amplified fragments were resolved by electrophoretic separation through a 1.2% agarose gel. The *Ttd1a* signals were normalized using the actin gene signals. Clones having inserts larger than 1,000 bp were sequenced.

Cloning and sequencing

The 1-kb PCR amplification products were purified from agarose gel and cloned into a pGEM-T Easy Vector system (Promega) by mixing 3 µl of amplified product with 50 ng of pGEM-T Easy Vector, 3 U T4 ligase, and 1× ligation buffer in 10 µl volume. The ligation product was cleaned with sec-butanol and precipitated with ethanol. The sample was resuspended in 10 µl of 0.5× Tris–EDTA and transformed into *Escherichia coli* cells. The clones were sequenced using ABI 377 automated DNA sequencer (Applied Biosystems).

Isolating RNaseH-LTR fragment

To isolate RNaseH-LTR sequences, we used the Pearce protocol (Pearce et al. 2000) with modifications, using specific primers targeted at the RT sequence. Partially restricted DNA (*Eco*RI for 2 h) was ligated to adaptors (Adat.1–Adat.2) overnight at 4°C. From a 10× dilution of the restriction–ligation reaction, 1 µl was taken as template for 20 µl of PCR, containing 12.5 pmol of RT primer, 12.5 pmol of *Eco*R1 adaptor primer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs and 1 U of Hotstar *Taq* DNA Polymerase (Qiagen). Cycling conditions were those specified by Pearce et al. (1999), with minor modification, consisting of a denaturation step at 95°C for 15 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension of 10 min. The amplified fragments were purified as described above and cloned using pGEM-T easy cloning kit (Promega). Positive clones were sequenced and analyzed using CENSOR software (http://www.girinst.org/censor) (Jurka et al. 1996).

RT-PCR analysis

RNA samples (2 µg) were reverse transcribed in a 20-µl reaction using the ThermoScript RT-PCR system for first-strand cDNA synthesis (Invitrogen). From this reaction, 3 µl of aliquots were amplified by PCR using the T.d.1 and T.d.2 primers. PCR amplification with *Taq* DNA polymerase was performed in a 30-cycle reaction under the following conditions: 94°C for 45 s, 56°C for 30 s and 72°C for 60 s.

Analysis in silico of the *Ttd1a* promoter region

Search for *cis*-elements in the *Ttd1a* promoter was performed using two software: the PLACE signal scan search provided by the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE; Higo et al. 1999) and the computer program provided by the PlantCare database (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html; Lescot et al. 2002). The analyses were performed with a 60–90% degree severity.

Sequence-specific amplification polymorphisms procedure (S-SAP)

The S-SAP procedure was performed as described by Waugh et al. (1997) with the following modifications. Total

genomic DNA (2 µg) from *T. durum* was digested with *Eco*RI (20 U) (Roche) restriction enzyme for 2 h at 37°C in a final volume of 50 µl. The digested DNA samples were incubated at 80°C for 20 min to inactivate the restriction enzymes. For ligation, 10 µl of the restriction reaction was added to a mixture containing 1 U T4 DNA ligase and 1 mM ATP in 10 mM of Tris–HCL (pH 7.5), 10 mM MgAc and 50 mM KAc, to which 40 pmol *Eco*RI phosphorylated adaptors were added. The reaction was incubated overnight at 4°C and then pre-amplified, using 12.5 pmol of ^{33}P-labeled LTR (PPT-LTR) primer and primer complementary to the adaptor sequences, with no selective nucleotide, namely *Eco*RI, in a final volume of 20 µl containing 12.5 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 1U of Hot-star *Taq* DNA Polymerase (Qiagen). The PCR conditions were as follows: denaturation step at 95°C for 15 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension of 10 min. Selective amplification was performed with primer pairs containing one of the selective nucleotides on the adaptor primers namely ******Eco*RI + A, *Eco*RI + C, *Eco*RI + G, *Eco*RI + T in combination with ^{33}P-labeled LTR (PPT-LTR) primer. The amplified fragments were separated on 6.5% polyacrylamide sequencing gels (20 × 30 × 0.04 cm). Samples were then run at 80 V for 18 h in 1× TBE and visualized by autoradiography.

S-SAP analysis was performed on three different biological samples, each one constituting 30 plants for each treatment (control, salt, light). New S-SAP bands that were clearly separated from non-polymorphic bands were selected, excised from gels using a razor blade and re-suspended in 100 µl of water. DNA was eluted from the bands by boiling for 15 min, salt-precipitated and resuspended in 10 µl of sterile water. A 5-µl sample was then used as DNA template in PCR amplification using the S-SAP conditions and primers described above. Amplification products were separated on 1.5% agarose gels, eluted, cloned and sequenced.

The transpositional nature of each new S-SAP band was tested by PCR. Primers (sequence available upon request) were designed from the flanking genomic regions of clones sequenced. Direct PCRs were performed using these primers and the LTR-7 primer. In each case, DNA from salt and light stresses, from which the new SSAP band was characterized, was tested against control DNA samples. A new insertion was indicated when a PCR product of the expected molecular weight was obtained with DNA from the corresponding salt and light stresses, but not with control DNA samples. All new insertions were named as follows: the letter indicates the type of stress (l = light and s = salt); the number identified the transposed copies of S-SAP band.

Sequence analysis

Sequence data were analyzed and compared to the GenBank-NCBI databases using the BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/) and GrainGenes (http://wheat.pw.usda.gov/GG2/blast.shtml). CLUSTAL W 1.8 program was used to generate most multiple sequence alignments (Thompson et al. 1994).

Results

Identification of a new family of retrotransposons in *Triticum durum*

Degenerate oligonucleotide primers RT1 and RNaseH1, designed from conserved motifs RT (DVKTA(T)FL) and RNaseH (T(S)KHIDVR) domains and derived from consensus sequences of *Tnt1* (Grandbastien et al. 1997), *Tto1* (Hirochika et al. 1996a, b), *Ta1*-3-like (Theologis et al. 2000) and *Silvia* (Guyot et al. 2005) retrotransposon elements, were used to amplify corresponding segments from wheat genome DNA by PCR. An amplification product of the expected size (approximately 1,000 bp) was obtained as visualized on 1.2% agarose gel. The amplified fragment was cloned into the pGemT vector and 20 positive clones were sequenced using ABI 377 automated DNA sequencer (Applied Biosystems). Based on a search against the GenBanck database, 12 sequences showed clear similarity to RT-RnaseH sequences, and their deduced amino acids showed significant homology to RT/RnaseH. Analysis of the obtained nucleotide sequences revealed multiple variations among clones, actually representing 12 different fragments of similar size. Of the 12 RT/RnaseH gene sequences, 11 were affected by either stop codons or translational frameshifts indicating that these clones could be transcriptionally inactive, while only one sequence of the wheat RT/RnaseH, which we named *Ttd1a*, was unaffected by stop codons or frameshifts when translated.

Isolation of *Ttd1a* LTR sequence from *T. durum*

Using the specific primers designed on the 3′ terminal RT gene (cloned *Ttd1a)* and restriction site-specific adaptors, we successfully amplified the putative Ty1-*copia* RNaseH-LTR wheat region. The fragments were cloned, and 24 clones were sequenced. The sequences were analyzed using CENSOR software (Jurka et al. 1996) and were individually compared with consensus RNaseH-LTR region (Pearce et al. 1999). In particular, seven positive clones were scanned for the following features: the conserved ART-KIEVD and ADIFTK RNaseH gene domains, a stop codon, a polypurine tract (PPT), an inverted terminal repeat (IR), and a complete putative 3′ LTR sequence (Fig. 1). We

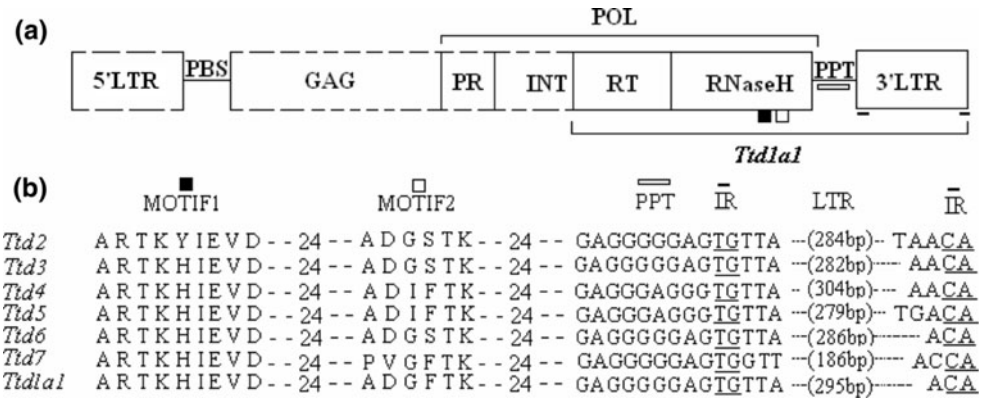

Fig. 1 **a** Structural feature of Ty1-*copia* retrotransposons: RNaseH, polypurine tract (*PPT*), inverted terminal repeat (*IR*) and terminal LTR sequences, **b** Peptide sequences of RNaseH and nucleotide sequences of PPT and 3′ LTR are aligned. The size of LTRs are indicated in *parenthesis*

named these sequences *Ttd, Ttd2, Ttd3, Ttd4, Ttd5, Ttd6* and *Ttd7*. The sequencing results showed that the *Ttd* clone was the extension sequence in 3′-flanking RT-region of *Ttd1a*. Our LTR sequence showed a very low similarity to other LTRs already present in gene Bank, and for this reason we gave it the new name *Ttd1a*. For instance, a search on the TREP Web site (http://wheat.pw.usda.gov/ITMI/Repeats/) that lists all currently known wheat retrotransposons shows that the first 300 bp of *Ttd1a* are 96% identical to RT domain of Angela retrotransposon, while the rest of the sequence shows no significant identity correspondence (i.e., <80%) to any other known wheat retrotransposons. This could be due to the existence of a chimeric wheat retrotransposon. We exclude that this is due to PCR recombinations or PCR artifact linking fragments of different origins. In this regard, we isolated the entire sequence (RT/RNaseH/LTR) using one step of PCR with specific primers designed to RT and LTR domain of *Ttd1a*. The result shows that the entire sequence is identical to that obtained using the methods described above (DOP-PCR and method of Pearce).

Expression analysis of the retrotransposon *Ttd1a*

The abiotic and biotic stresses are known to trigger the expression of a wide range of genes related to plant defense responses. Here, we examined *Ttd1a* transcript in leaves of *T. durum* by RT-PCR analysis. Because some plant retrotransposons are known to be transcriptionally activated by stress, we decided to investigate the action of salt and light stresses on the transcriptional level of *Ttd1a*. As control, RT-PCR reactions designed to amplify a 500-bp region of the actin gene were performed (P1–P2 primers). As shown in Fig. 2, an amplified product (approximately 1,100 bp) corresponding to the expected size of the *Ttd1a* RT and U3 partial cDNA region (Td1–Td2 primers) was detected only in plants grown under salt or light stresses, without any detectable expression in control plants. No changes in the transcripts on stress treatments were observed in the housekeeping actin gene from wheat. The negative control was

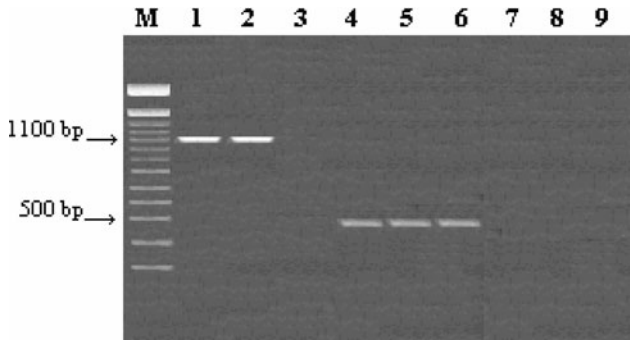

Fig. 2 RT-PCR analysis of *Ttd1a* transcription in *T. durum* genome. *Lanes*: *M* molecular mass markers (Invitrogen), weight size markers are indicated on the *left* and the correct PCR product sizes on the *right*; *1 Ttd1a* cDNA from salt-stressed leaves; *2 Ttd1a* cDNA from light-stressed leaves; *3 Ttd1a* cDNA from control leaves; *lanes 4–6*, amplification of actin gene (control reactions) in salt-stressed, light-stressed and control plants, respectively

performed to demonstrate the absence of DNA contamination in RNA samples, from three different treatments (control, salt and light). The RT-PCR products obtained from both salt and light-stressed plants were cloned and sequenced. Sequence analysis showed that all clones were identical to *Ttd1a*. These results suggest the presence of specific sequence motifs acting as regulatory elements that modulate the transcriptional activation of the *Ttd1a* in response to salt and light stresses.

In silico analysis of potential *cis*-elements in the *Ttd1a* promoter region

To identify the putative *cis*-regulatory elements participating in the activation of the *Ttd1a* element under salt and light stresses in the U3 putative region, we analyzed its nucleotide sequence using two databases: PlantCARE (http://www.bioinformatics.psb.urgent.be/webtools/plantcare/html; Lescot et al. 2002) and PLACE (http://www.dna.affre.go.jp/htdocs/PLACE/; Higo et al. 1999). Analyses were performed by diminishing the degree of severity to 60–90%. *cis*-Acting motifs contained in the *Ttd1a* LTR

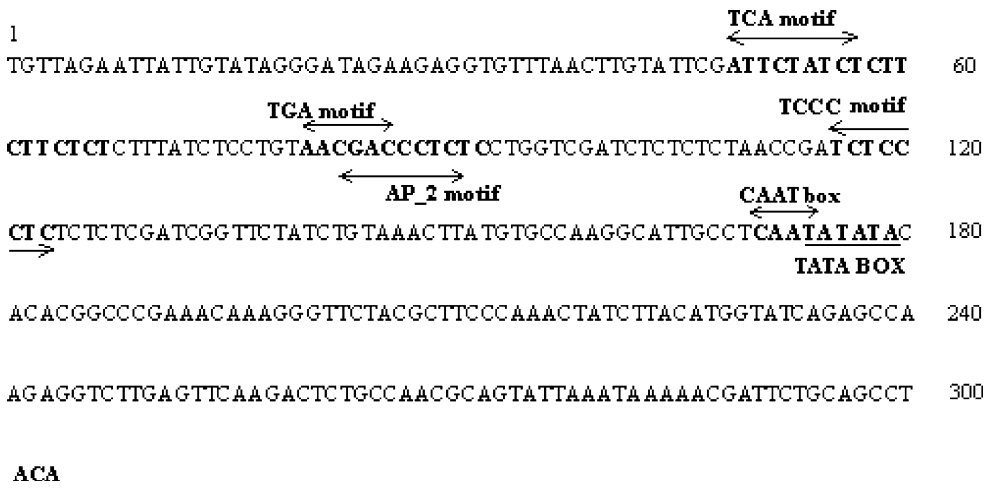

Fig. 3 Nucleotide sequence of 3′ LTR of *Ttd1a* retrotransposon: U3 domain (nt 1–210), R domain (nt 211–218), U5 domain (nt 219–303). The box motifs are in blot and indicated by *double arrows*. The putative TATA promoter elements are *underlined*

Table 1 Promoter motifs identified in the U3 domain of *Ttd1a* retrotransposon

Motif	Organism	Matrix score	Sequence	Function
CAAT-box	*Hordeum vulgare*	6	CAAT	Common *cis*-acting element in promoter and enhancer regions
TCA-element	*Brassica oleracea*	9	GAGAAGAATA	*cis*-acting element involved in salicylic acid responsiveness
TCCC-motif	*S'pinacia oleracea*	7	TCTCCCT	Part of a light-responsive element
TGA element	*Brassica oleracea*	6	AACGAC	Auxin-responsive element
Plant_AP-2-like	*Oryza sativa*	8	CGACCAGG	Genes that encode proteins involved in the regulation of disease-resistance pathways

region share high similarities with the motifs involved in the activation of several plant defense genes (Fig. 3; Table 1). CAAT motif (nt 171–174) is present in the regulatory regions of several plant defense genes and involved in the expression of the HVA1 gene of barley (*Hordeum vulgare*) (Ross and Shen 2006). The TCA motif (nt 57–48, in the complementary strand) is highly conserved among stress-inducible genes and binds salicylic acid-inducible nuclear proteins (*Brassica oleracea*) (Pastuglia et al. 1997). Finally, TCTCCCT, AACGAC and CGACCAGG sequences showed respective homology to (1) the TCCC (nt 116–123) conserved in the plastocyanin (PC) gene promoter from spinach (Bichler and Herrmann 1990), (2) the TGA motif (nt 81–86) present in the S gene family promoter regions from *B. oleracea* (Pastuglia et al. 1997), (3) the AP_2 motif (nt 92–84; in the complementary strand) (Gutterson and Reuber 2004) showing a binding site for a AP2 transcription factor family (Fig. 3; Table 1).

Ttd1a LTR-derived S-SAP in plant of wheat grown under salt and light stresses

The S-SAP procedure was developed to analyze *Ttd1a* new insertion polymorphism in durum wheat grown under salt and light stresses. Since the LTR sequences of Ty1-*copia* retrotransposons are present at both ends of the retrotransposon and are identical, we used a 22-bp primer PPT-LTR, which included the 6-bp terminal sequence of the RNaseH region, the entire PPT (9 bp) and the 7-bp initial sequence of 3′LTR of the *Ttd1a* orientated toward the 3′ end, to prevent unspecific amplification of the internal region coding GAG gene of 5′ retrotransposon. The S-SAP analysis was performed using a single selective base on the *Eco*RI primer in combination with an LTR-specific primer and it showed new S-SAP bands (Fig. 4).

It is noticeable that new S-SAP bands were detected in a reproducible manner in an S-SAP experiment performed with DNA isolated from three different biological samples, of which each one constituted a pool of 30 plants for every treatment (control, salt and light) (data not shown). Furthermore, SSAP experiments were repeated three times and identical patterns were observed in control samples, indicating the absence of interplant polymorphism. Each new insertion identified in a sample came from 30 plants (under stress) involving insertional events in a single plant and not in all 30 plants, because the probability that two or more events occur at the same integration site is practically zero. We used a pool of 30 plants to increase the probability of identifying new transpositional events.

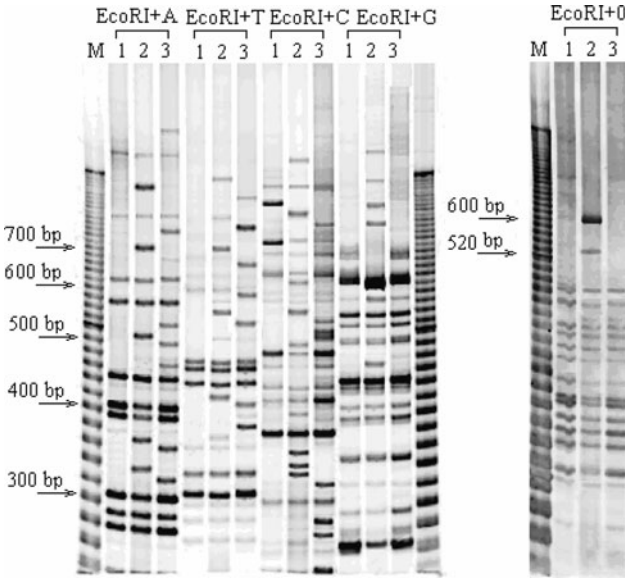

Fig. 4 S-SAP profiles of *Ttd1a* retrotransposon with different selective primer combinations: PPT-*Eco*RI + A; PPT-*Eco*RI + T; PPT-*Eco*RI + C; PPT-*Eco*RI + G; PPT-*Eco*RI + 0. *Lanes*: *M* mass molecular ladder, *1, 2, 3*, genomic DNA from control, salt-stressed and light-stressed plants, respectively

Each combination of S-SAP primers involved the *Ttd1a*-derived primer in conjunction with one of four selective *Eco*RI primers generating different S-SAP profiles for both salt and light stresses. Each new S-SAP amplification band therefore concatenates a region at the 3′LTR of the target retrotransposon and the unique 'host' genomic sequence in which each *Ttd1a* retrotransposon copy is embedded. Control S-SAP experiments were performed on plants grown in control conditions. The *Eco*RI primer combination with no selective bases on the 3′ end generated weak S-SAP profiles compared with the *Eco*RI primer combinations that contained a selective base. This may be due to the competition between the different copies of *Ttd1a* in the PCR. In the experiments run on leaves of wheat plants grown under salt stress, new S-SAP bands were detected with all four combinations of primers, each one containing a selective base. This combination gave a good and clear S-SAP marker profile, with excellent band reproducibility. These results implied that *Ttd1a* existed in the wheat genome in high copy numbers.

Using *Eco*RI + T, *Eco*RI + A *Eco*RI + C primers in light stress experiment, new S-SAP bands were detected in lower numbers. This combination produced overall polymorphic bands. Other combinations of primers did not produce new bands. Slightly smaller products (approximately 280 and 260 in size) were amplified with the *Eco*RI + C primer under light stress. This product could be due to a subclass of *Ttd1a* element with an *Eco*RI site closer to the primer (LTR) than the main 303-nucleotide product.

A total of 23 new S-SAP random bands (13 from salt stress, 10 from light stress) were eluted and sequenced after re-amplification from gel. The transpositional nature of each new S-SAP band was tested by direct PCR performed using the LTR-7 primer and primers designed in each flanking genomic sequence. The analysis showed that only 13 of the 23 new bands selected corresponded to transposition events. The remaining ten bands, obtained from salt and light stresses, could not be identified as transposed copies, suggesting that the failure to identify the transposed copy represents the background of technical problems inherent in our cloning strategy.

All the newly transposed copies analyzed belong to the *Ttd1a* group (Fig. 5). Moreover, five of the newly transposed copies are identical to the *Ttd1a* sequence. The remaining eight transposed copies show small variations in sequences compared to the *Ttd1a* LTR region (Fig. 5). The mutation within this region could affect the transcriptional activity of the different *Ttd1a* elements.

To determine the site of new insertions, each flanking sequence was used as a query in GrainGenes (http://wheat.pw.usda.gov/GG2/blast.shtml) searches of sequences database. Analysis of flanking sequences revealed that one new transposed copies of *Ttd1a* element is located in close proximity to the identified gene, while the other sequences belong to repetitive regions. These repetitive regions showed strong similarities to *Gypsy* and Ty1-*copia* retroelements of previously identified wheat genome. Our results are summarized in Table 2.

Discussion

Transcriptional induction of *Ttd1a* in response to salt and light stresses

In this study, we have isolated a new Ty1-*copia*-like retrotransposons from wheat genome using a PCR strategy to clone fragments of the RT and RNaseH genes. In agreement with high levels of heterogeneity observed among the RT genes of Ty1-*copia* group retrotransposons from plants (Kumar and Bennetzen 1999), all 12 clones differed from each other. This is consistent with the common understanding that Ty1-*copia* group retrotransposons pervade as large and highly heterogeneous populations as the entire plant kingdom (Flavell et al. 1992). Retrotransposons are mostly quiescent during development, but become active under stress conditions. We also have shown that transcription of *Ttd1a* is induced when *T. durum* plants are exposed to abiotic stresses.

Several stresses are known to increase the transcriptional levels of plant retrotransposons (Grandbastien 2004; Hirochika et al. 1996a; Picault et al. 2009; Takeda et al. 1999), but up to now, no retroelements have been shown to be transcriptionally activated by visible light. An increase in the expression of retrotransposons by UV light irradiation

Fig. 5 Sequence variability of the LTR sequence of new *Ttd1a* copies characterized in plants treated with salt and light stresses. Sequences have been aligned on the *Ttd1a* element. *Dashes* and *blanks* indicate sequence identity and deletions, respectively. Nucleotides that differ from the reference sequence are shown in *capital letters*

Table 2 GrainGenes database search for determining the nature of the *Ttd1a* insertion sites

Transposed copies	Type of stress	Nature flanking sequence	Size of flanking (bp)	Identity (%)
12	Light	Known TE: Ty3 gypsy Erika inserted in gag region	823	94
14	Light	Known TE: Ty3 gypsy SUKKULA inserted in pol region	347	91
17	Light	Known TE: Ty3 gypsy SUMAYA inserted in gag region	337	93
111	Light	Known TE: Copia-type AngelaB inserted in gag region	750	90
113	Light	Known TE: Copia-type IDA inserted in gag region	651	89
s2	Salt	Known TE: Copia-type WisB inserted in gag region	648	93
s3	Salt	Known TE: Copia-type Angela inserted in gag region	550	98
s5	Salt	Known TE: Copia-type Daniela inserted in 5′ LTR region	400	94
s6	Salt	Known TE: Copia-type Eugene inserted in 5′ LTR region	551	95
s7	Salt	Near CDS: putative resistance protein RGA2. Inserted 1,855 bp upstream from ATG	650	92
s8	Salt	Known TE: Ty3 gypsy SUMAYA inserted in 3′ LTR region	765	90
s13	Salt	Known TE: Copia-type Maximus inserted in 3′ LTR region	350	96
s19	Salt	Known TE: Copia-type Claudia inserted in pol region	606	94

has been observed for SINEs in mammals (Valerie et al. 1996) and some LTR retroelements in animals (Shim et al. 2000), yeast (Sacerdot et al. 2004) and plants (Kimura et al. 2001; Ramallo et al. 2008).

Plants inherit different adaptive mechanisms to acclimatize to abiotic stresses, which culminate in the expression of a wide spectrum of stress-responsive genes and gene products. Studies in model plants suggest that different

biochemical pathways share common elements that are associated with adaptation to a range of abiotic stresses (Knight and Knight 2001).

In silico analysis of the sequence of the *Ttd1a* promoter showed that U3 putative region contained five putative *cis*-acting elements including CAAT, TCA, TCCC, TGA and AP_2 motifs (Fig. 3). These putative transcription factor binding sites displayed highly similar sequence to motifs involved in the transcriptional activation of defense genes in plants (Bichler and Herrmann 1990; Gutterson and Reuber 2004; Pastuglia et al. 1997; Ross and Shen 2006). They also showed high conservation in the sequence core, which is the most important region for annealing of transcription factors. Similar regulatory sequences, present in the promoters of defense genes and in the U3 domain of *Ttd1a* retrotransposon, could be involved in processes such as the signaling cross talk of metabolite and environmental stress and confer a selective advantage either to the host plant, such as through stress adaption, or to the element itself. Study of *Tto1* and *Tnt1* retrotransposon promoters in tobacco genome (Casacuberta and Grandbastien 1993; Takeda et al. 1999) showed the presence of *cis*-regulatory elements similar to those involved in the regulation of endogenous genes when the plant is under stress.

The activation of *Ttd1a* by salt and light stresses, which are the same as those involved in the activation of plant defense responses, is intriguing. However, the biological significance, as well the origin of regulatory sequences in retrotransposons and host genes, is unclear at present and warrants further investigation. In fact, these motifs are highly variable and generally depend on the secondary structure of DNA for their specificity as promoter signal in a sequence. It would be necessary to determine and validate the role of these *cis*-acting elements in the stress activation of *Ttd1a* by performing promoter deletion analysis using a reporter gene.

Ttd1a mobility by salt and light stresses

Using S-SAP strategy, we have investigated the transposition of *Ttd1a* retroelement and its insertional polymorphism in the wheat genome. The results show that light and salt stresses are able to induce *Ttd1a* mobility and to generate genetic diversity in durum wheat plant genome. In fact, newly transposed copies of *Ttd1a* were detected only in wheat plants grown under stress conditions. Many studies have shown insertion polymorphisms of several elements, and the use of the S-SAP strategy to detect and quantify the mobility of retrotransposons has also been shown in other species. Pearce et al. (2000) developed an S-SAP strategy for *PDR1* retrotransposon in pea to study the transpositional activity. Melayah et al. (2001), Grandbastien et al. (2005) and Le et al. (2007) developed an S-SAP strategy to evaluate and monitor the impact of stress on *Tnt1* mobilization in tobacco plants regenerated from mesophyll leaf cell, either explants culture, via protoplast isolation and exposure to fungal extracts.

We compare the *Ttd1a* insertions-polymorphism in durum wheat plants grown under salt and light stresses, respectively. Both stress conditions triggered the mobility of *Ttd1a* and, in particular, salt stress was more efficient for *Ttd1a* transposition than light stress, while no transposed copies were observed in plants grown in physiological condition.

We assumed that the high efficiency of *Ttd1a* mobilization in our plants grown under salt stress was attributable to the stronger effect of salt stress on the defense response of plants. In fact, salt stress-tolerant plants have evolved different adaptive mechanisms to display different degrees of tolerance, which are largely determined by genetic plasticity.

Profiling of new multiple *Ttd1a* insertions were found in plants grown under stress conditions (Fig. 4) and results are summarized in Table 2. The high polymorphisms observed in both types of stress may be the results of the presence of different subfamilies of *Ttd1a* element. This hypothesis is supported by the presence of smaller products (less of 300 bp) and, as *Ttd1a* LTR sequence does not contain sites for the restriction enzyme *Eco*RI, such products may be due to a member of the *Ttd1a* family (Fig. 4). The high level of *Ttd1a* transposition observed in our experiments open the perspective to assess efficiently the genetic impact of stresses such as salt and light stresses applied on plants.

Kashkush and Khasdan (2007) used a novel technique called transposon methylation display (TMD) to study the methylation patterns of TEs and their flanking sequences in different tissues. In their study, Kashkush and Khasdan showed that tissue-specific LTR methylation correlated with tissue-specific expression of the flanking rice gene. It is known that methylation patterns change in a tissue-specific manner (Khodosevich et al. 2004; Lavie et al. 2005) and that TE methylation status has been shown to alter the methylation of flanking host sequences during development (Khodosevich et al. 2004).

In our study, new bands observed under stress in the SSAP experiments were not due to epigenetic factors (*Eco*RI site methylation) because all bands present in control samples also appeared in stress samples with the exception of one band using the PPT-LTR/*Eco*RI + C combination primer (Fig. 4).

We examined the same sequences flanking newly transposed *Ttd1a* insertions, which were recovered from S-SAP profiles of wheat plants grown under stresses. Using sequence similarity research, we noticed that new transposition events appear to be preferentially inserted in the retroelements belonging to Ty3-Gypsy and Ty1-*copia* family.

This is not surprising considering that in the wheat genome, the coding region represents a small fraction of the total DNA.

In cereal genomes, retrotransposons are associated in very large intergenic spacers, where elements are nested within each other (Suoniemi et al. 1996). Nested insertions reduce active copy number, because the so-formed multimers cannot fully transpose. Insertion element *Ttd1a* in other retrotransposons could be attributed to the self defense mechanisms, giving some selective advantages to the host plant.

Although the meaning of nested insertion is not understood, one possible explanation is that targeting retrotransposons into non-essential retrotransposonic sequences helps to minimize deleterious mutations resulting from transposition. Selection of the insertion site may be made by an integration complex, consisting of retrotransposon-encoded integrase, a reverse-transcribed cDNA copy of the element, and various host-encoded factors (Sabot and Schulman 2006). However, preference for insertion into sequences target in their host may be due to an explicit interaction between feature of LTR retrotransposons and natural host selected during evolution. In fact, plants have acquired different sets of metabolic processes to remove and/or inactivate different populations of TEs (Bennetzen 2000).

Interestingly, one new insertion (FN432153) was found upstream to the start codon (1,855 bp) of disease resistance RGA2 gene belonging to the family of CC-NBS-LRR protein (AY663391). This protein confers resistance to a broad spectrum of pathogens, including viruses, bacteria, fungi and nematodes (McHale et al. 2006). Transposition in the vicinity of identifiable gene underscores that stress-induced transposon activity can potentially modulate the function of cellular genes. Insertions within coding region may be better tolerated in polyploid plants because polyploidy provides extra gene copies that can compensate for any allelic deficiencies resulting from a mutational insertion. Furthermore, homologous copies of a gene mutated by retrotransposon insertions can provide a normal expression pattern, although there may be a dosage dependent effect. Alternatively, insertion in coding regions could be subsequently counter-selected by evolutionary processes, while insertions in repetitive DNA are maintained and gradually accumulate.

In the rice genome, Hayashi and Yoshida (2009) reported that an LTR retrotransposon named Renovator contributed to the evolution of the resistance gene *Pit,* which conferred race-specific resistance against the fungal pathogen *Magnaporthe grisea*. Their results suggested that transposon-mediated transcriptional activation might play an important role in the refunctionalization of additional "sleeping" R genes in the plant genome.

The presence of fragments or complete retrotransposon copies within the regulatory regions of genes indicates that they have evolved into a form that is involved in specific gene regulation (Kumar and Bennetzen 1999). Studies conducted by Yan et al. (2006) on wheat genome have demonstrated that the dominant *Vrn3* allele was associated with the insertion of a retroelement in the *TaFT* promoter and that, in winter, wheat plants transformed with the *TaFT* allele carrying the promoter retroelement insertion flowered significantly earlier than non-transgenic plants.

When an element transposes into a new site, it can destroy or alter the expression pattern of coding genes (Kumar and Bennetzen 1999) and its insertion in intergenic regions can also modify the expression of adjacent genes with changes in host fitness. Examples of associations between retrotransposon and plant genes, as well as of their impact on gene structure and function and potential use as promoter sequence, have been found (Hayashi and Yoshida 2009; Le et al. 2007; Lenoir et al. 2001; White et al. 1994). It has been recently shown that inter-specific hybridizations can reactivate the transcription of wheat WIS2 retrotransposons, which can drive the readout synthesis of new transcripts from adjacent sequences including sense and antisense strands of genes located nearby, resulting in the silencing or the activation of these genes (Kashkush et al. 2003).

Ttd1a activation might be a "form of evolutionary conservation" in wheat genome by providing new properties through amplification mechanisms in response to environmental stresses. It could be of interest to understand if these neo-insertions come through the descent of these plants, and if so what is the true evolutive impact on the species. It is known that leaves derived from shoot apical meristems (SAMs) and in cereal crops, SAM are constituted from two cell layers (L1 and L2) in continuous division (Ahmad et al. 2002). L2 is especially important in stable genetic transformation because it is the layer that will develop into the reproductive structures that form the gametes, which will pass the genetic material onto subsequent generations (Chandra and Pental 2003). Leaves typically originate from both the layers, but sometimes from only the L2 layer (Lyndon 1998). *Ttd1a* neo-insertions observed in young leaves could be transmitted to the offspring only if this mobilization occurs in the L2 layer cells.

Our results represent the first direct demonstration that salt and light stresses, known to activate plant defense responses, are also able to generate new polymorphisms through retrotransposon mobilization. Our study, therefore, improves the hypothesis that stress modulation of retrotransposons might play a role in generating host genetic plasticity in response to environmental stress (McClintock 1984).

The background information on the TE sequences could allow the construction of natural carriers that act as strong promoters, which are capable of modeling the gene expression.

This type of construct is also of potential use to test the response of different plant species to abiotic and biotic stress. To study more thoroughly the contribution of the 5′ LTR promoter region into the transcription of wheat *Ttd1a* retrotransposon and to clarify the question of its role as an additional promoter to promoter stress-response genes, it is necessary to analyze the expression by a reporter construct. Further studies are needed to better understand of the complex and interesting issues of the role that mobile elements play in the structure and evolution of host genomes.

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