miRNA-guided cleavage of the transcript is usually required for phased ta-siRNA biogenesis [2, 5, 6, 10]. At this point, Suppressor of Gene Silencing 3 (SGS3) protects the ssRNA from degradation. Next, RNA-dependent Polymerase 6 (RDR6) produces a complementary strand, turning the transcript into double stranded RNA (dsRNA) until non-cleaved site of TAS3 transcript bound to AGO-miRNA comples [6, 10]. Dicer-like Protein 4 (DCL4) then cleaves the dsRNA in 21-nt increments to generate mature ta-siRNAs [4, 6, 10].

The TAS3 gene-mediated ta-siRNA pathway is a plant-specific miR390-dependent phenomenon conserved in all higher plants and in primitive land plants such as *Physcomitrella* and other mosses [6, 9]. In *Arabidopsis*, miR390-guided cleavage occurs only at the 3′ target site of the TAS3 precursor where canonical targeting rules include perfect complementarity between miRNA nucleotides 2–13 (from the 5' end) and the mRNA target [6] (Fig. 1). The 5′ target site is resistant to cleavage but is important for processing of the tasiRNA precursor transcript. This characteristic feature is attributed to mismatches at nucleotides 9–12 from the 5′ end of the miRNA (Fig. 1). These mismatches are highly conserved in the higher plant TAS3-primary transcripts [6, 8].

In this paper, we have studied the presence of tasiARF RNA genes in the representatives of genus *Senecio*having either unifacial or true bifacial leaves. It was found that both types of species encode such RNA precursors principally similar to those found in Arabidopsis. We used primers mimicking miR390 (Krasnikova et al., 2009) and oligo (dT)-based oligonucleotides. One of the TAS3 species (TAS3-Sen1) in Senecio representatives was actively transcribed and distributed among many *Asteracea* plants and found to be similar to AtTAS3a. The data obtained may contribute to understanding of expression regulation of *Senecio* TAS3-like genes and the potential involvement of these RNAs in development of plant organ abaxial-adaxial symmetry.

Materials and Methods. *Plant material*. Specimens for *Senecio talinoides, Curio repens* and *Curio articulatus* were taken from collections of the N.V. Cycin Main Botanic Garden of the Russian Academy of Sciences. *Analysis of Nucleic Acids.*Genomic plant DNA was isolated from 200 mg of plant material by DNA extraction kit (Macherey-Nagel) according to the protocol of the manufacturer. TAS3 genes were amplified and sequences as described in [11]. Total RNA was isolated from *Nicotiana benthamiana* leaves with the Trizol reagent according to the manufacturer's instructions (Invitrogen). Digestion of any contaminating DNA was achieved by treatment of samples with RQI RNase-free DNase (Promega). Reverse transcription was performed with 1 *μ*g of total RNA and oligo (dT)-primer t20-xho (attctcgaggccgaggcggccgacatgttttttttttttttttttttttttttv) using the RT system (Invitrogen) according to the protocol of the manufacturer. Primers for dicotyledonous plants were: forward primer: TAS-P (5'-GGTGCTATCCTATCTGAGCTT-3')and mixture of reverse primers TAS-Mcaa (5'-AGCTCAGGAGGGATAGCAA-3')and TAS-Maca (5'-AGCTCAGGAGGGATAGACA-3'). For PCR, 25–35 cycles were used for amplification with a melting temperature of 95°C, an annealing temperature of 58°C and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 3 min. PCR products were separated by electrophoresis of samples in 1.5% agarose gel and purified using the GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences). For cloning, the PCR-amplified DNA bands isolated from gel were ligated into pGEM-T (Promega). Cloned products were used as templates in sequencing reactions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA sequences were deposited at the NCBI data bank, the accession numbers are JN692262, JN692261, JN692260 and JN692259. The TAS3 cDNA sequences were cut out of the vectors pGEM-T by digestion with HindIII and ligated into HindIII-digested binary vector pLH7000 containing the flanking 35S promoter and transcriptional terminator and provided by Dr L. Hausmann (Federal Centre for Breeding Research on Cultivated Plants, Germany). Agroinfiltration of *Nicotiana benthamiana* plants was carried out as described [12]. RNA was isolated 4 days after agroinfiltration of plant leaves (see above).

Results and Discussion.*Identification and molecular phylogeny of the most abundant TAS3-like species in representatives of family Asteraceae.*Leaves of angiosperms exhibit considerable morphological diversity and thus represent an attractive subject for developmental molecular studies [13]. The diverse leaf forms in angiosperms can be categorized as bifacial or unifacial. Bifacial leaves, such as those of Arabidopsis thaliana, are the more typical form of leaves that differentiate adaxial-abaxial (upperlower) polarity. The establishment of adaxial-abaxial polarity in bifacial leaves is regulated by overlapping and antagonistic genetic interactions involving several distinct transcription factors and small regulatory RNAs [14]. Unifacial leaves, which are characterized by an abaxialized leaf blade, have repeatedly evolved in a number of divergent monocot species [15]. Transcription factors *ARF3* and *ARF4* are required for specifying leaf abaxial identity [14]. On the other hand, *ARF3*/*4* transcripts are targets of a *TAS3*-derived *trans*-acting short interfering RNA, tasiR-ARF, which guides the cleavage of *ARF3/4* mRNAs (see above). The *Arabidopsis* TAS3 is expressed on the adaxial side of early leaf primordia, which possibly regulates the expression of ARF3 and ARF4 in the adaxial domain and determines the dorso-ventral leaf polarity [16]. Genetic analyses in maize and rice have also demonstrated a primary role for ta-siARF during dorsiventral leaf patterning [17, 18]. To further study the role of TAS3 ta-siRNA in development of leaf polarity in dicot plants, we started the studies of some representatives of family *Asteraceae* where some succulent plants of Senecioneae are characterized by an abaxialized unifacial leaf blade [19].

The *Asteraceae* is the second largest family of flowering plants with at least 1100 genera and over 20,000 species included. The *Asteraceae* includes shrubs and a few trees. Senecioneae is the largest tribe of *Asteraceae*, comprised of ca. 150 genera and 3,000 species. Approximately one-third of its species are placed in *Senecio*, making it one of the largest genera of flowering plants. Despite considerable efforts to classify and understand the striking morphological diversity in Senecioneae, little is known about its intergeneric relationships. This lack of phylogenetic understanding is predominantly caused by conflicting clues from morphological characters, the large size of the tribe, and the absence of a good delimitation of *Senecio*. Phylogenetic analyses of nuclear ribosomal ITS and plastid DNA sequence data were used to produce a hypothesis of evolutionary relationships in Senecioneae [20].

Previously, we described the new method for identification of plant ta-siRNA precursor genes based on PCR with oligodeoxyribonucleotide primers mimicking miR390. The method was found to be efficient for dicotyledonous plants, cycads, conifers and mosses [9, 11]. In this study, PCR-based approach was used as a tool to probe genomic DNA samples derived from three species of Senecioneae: *Senecio talinoides* with abaxialized unifacial leaves, *Curio repens* with pseudo-unifacial leaves and *Curio articulatus* with bifacial leaves [19]. PCR amplification of chromosomal DNA from these plants resulted in synthesis of one major band of 270 bp (data not shown). Cloning and sequencing

of the obtained DNA bands revealed that the amplified sequences are closely related (more than 90% identity) (data not shown) contained putative ta-siARF site composed of two tandem copies of ARF-specific ta-siRNAs and located between miR390 target sites corresponding to PCR primers (Fig. 1). This TAS3-like gene was named as TAS3-Sen1 (GenBank accession number – JN692259).

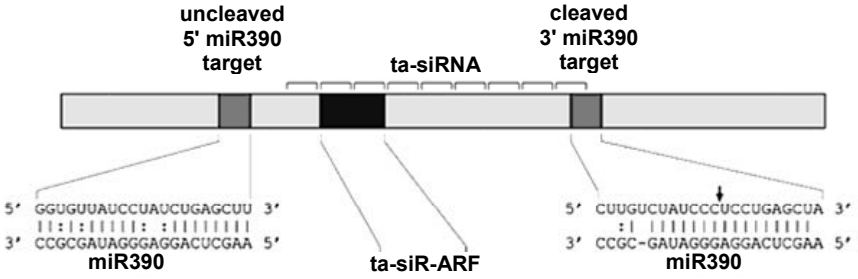

Figure 1. Organization of TAS3 tasiRNA precursors. The miR390-guided cleavage site is indicated by the arrow. The tasiRNA region is indicated by brackets.

Bioinformatic analysis of the putative TAS3-like sequences from *Senecio talinoides, Curio repens* and *Curio articulatus* using NCBI Blast revealed closely related sequences in other representatives of the family *Asteraceae* (data not shown and Fig. 2). Using available sequence databases, more distant TAS3-like sequences were identified at least in 2 families belonging to subclass Rosids and 11 families of flowering plants belonging to subclass Rosids, for which complete sequences of TAS3 cDNAs were revealed (see Materials and Methods; data not shown and Fig. 2). The phylogenetic tree based on comparisons of TAS3-like sequences demonstrated that all TAS3-Sen1 sequences of *Asteraceae* form a monophyletic group (Fig. 2). On the other hand, the closest branching lineage in eudicots was represented by plants from subclass Asterids, namely,orders Lamiales (*Antirrhinum majus*) and Solanales (*Nicotiana tabacum* and *Solanum tuberosum*) (Fig. 2). These observations are in agreement with the branching order of flowering plant evolution trees published previously [21].

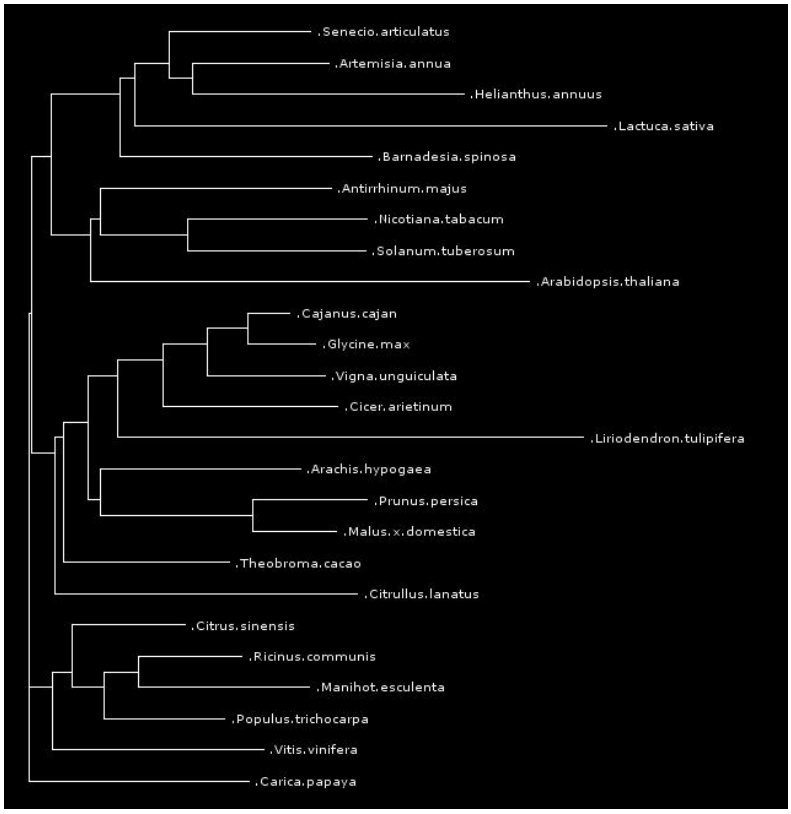

Figure. 2. The minimal evolution phylogenetic tree based on analysis of the aligned TAS3 genes from some flowering plants. This tree was generated according MAFFT6 program (http://mafft.cbrc.jp/alignment/server)

The TAS3-Sen1 family like other other TAS3 loci is characterized by the dual miR390 binding sites, which are functionally required by TAS3 mRNA to define the phasing register for tasiRNA production [6]. We conducted an exhaustive Blast search using TAS3-Sen1 genes as queries to find additional putative Senecio TAS3 genes in the NCBI databases. The *Senecio madagascariensis* cDNA contig (GenBank accession number SRR006592) includes the tasiARF region and flanking 5'-terminal miR390 complementary site. However, this cDNA contig was shorter than

the full-length TAS3 mRNA precursor and 3'-terminal miR390 site could not be mapped. Nevertheless, we used sequence of contig SRR006592 to reconstruct the authentic sequence (not derived from PCR primer mimicking miR390) of 5'-terminal miR390 complementary site (Fig. 3A). Therefore, the presence of miR390 complementary sites in the TAS3-Sen1 loci supported the idea that they were indeed tasiRNA genes, and suggested that miR390 set the phasing register of tasiARFs (Fig. 3A). Indeed, ta-siARF sequences in TAS3-Sen1 coaligned with the phases D7(+) and D8(+) defined by the 3′ miR390 processing site in their putative precursors as observed in angiosperms (Figs. 1 and 3A). [6, 22].TAS3-Sen1 loci in S.articulatus and S.madagascariensis both had identical region including two closely related 21-nt sequences adjacent to one another of which one was identical to Arabidopsis tasiARF at phase 5′D8(+) and the other contained three mismatches with respect to the Arabidopsis tasiARF at position 5′D7(+) (Fig. 3B). To verify whether the interaction between Senecio miR390 and the TAS3-Sen1 precursor is similar to what was observed in *Arabidopsis*, sequence comparison was performed to predict cleavage events at the 5′ and 3′ target sites on the TAS3 precursor. Canonical targeting rule including nearly perfect complementarity between miR390 nucleotides 2–13 (from the 5' end) and the TAS3 RNA 3'-terminal miR390 target was confirmed (Fig. 3). These results are also consistent with the previous suggestion that miR390 is unable to guide for a cleavage because of mismatches between 9 nt and 11 nt from the 5′ end of the miR390 [6, 22] (Fig. 3).

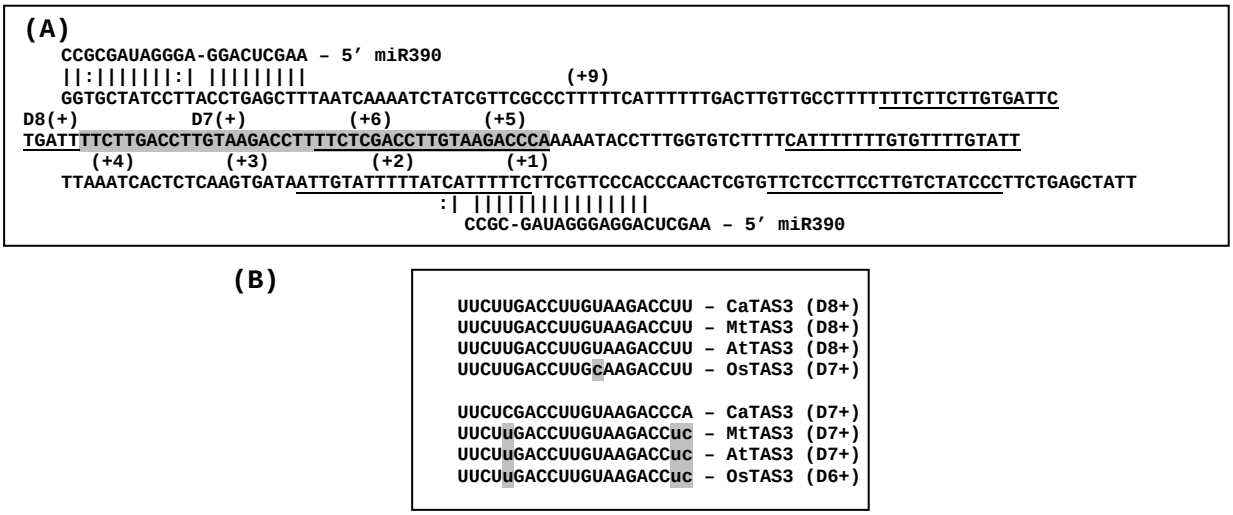

Figure 3. (A) Nucleotide sequence of TAS3-Sen1 locus. The 5′ and 3′ miR390 target sites are shown as alignments. Predicted Dicer processing sites are shown by underlining. TAS3-Sen1 siRNAs that are complementary to the auxin response factors are indicated by shading (+D7TAS3 and +D8TAS3).
(B) Sequence alignment of the TAS3-Sen1 and TAS3 ta-siARF RNAs from Arabidopsis, rice and *M. truncatula*

Cloning and sequencing of the TAS3-Sen1 cDNA species suggests a new strategy for the involvement of non-cleavable miR390 target site in control of ta-siARF formation. Processing and polyadenylation of plant TAS3 gene precursors has not been studied in detail excepting splicing of Arabidopsis thaliana gene TAS3a (AT3G17185) [23]. We have studied the polyadenylation sites of TAS3-Sen1 genes in three distinct Senecio species by sequence analysis of multiple cDNA clones. Figure 4 shows the nucleotide sequence of the 3' tail regions following ta-siARF tandem region of TAS3-Sen1 gene in three plant species and the polyadenylationsites of the analyzed cDNA clones. The cDNA clones for the genes of *S. talinoides, C. repens and C. articulatus* fall into groups with differentpolyadenylation sites. The two putative full-length St. 41 and Ca. 990 cDNA clones analyzed also had different poly A sites. Since the poly A tails of all the cDNA clones analyzed do not occur in the regions corresponding to a long AT rich area in the genomic sequence we are confident that correct priming occurred with the oligo dT during the cDNA preparation, and that the different groups of cDNA clones represent true differences in the poly A addition site on the mRNA's. In the case of Ca.91, St.33 and St.33 species many cDNA clones have been revealed for each gene. Eight out of 12 of the cDNA clones for *C. repens* and 7 out of 13 clones for *C. articulatus* had the poly A tail added in this middle position. The sites where polyadenylation occurs in the mRNA are spread over quite large distances (Fig. 4 and data not shown). The two polyadenylation sites detected for *C. articulatus* are 216 nucleotides apart, those for *S. talinoides* are separated by 269 nucleotides; however the three sites were detected for *C.* repenst. A high degree of variability in the positionning of the poly A tails therefore occurs between these highly related genes in three plant species. One feature which is common to Cr.29 and Cr.31 species is that the nucleotide in the genomic sequence which corresponds to the first A in the poly A tails of the cDNA clones, is an A residue. Also illustrated in Figure 4 is sequence in St.41 which may represent the G/A cluster sequences positioned immediately upstream of the poly A addition site. The potential importance of homopurine-homopyrimidine sequences in biological processes is suggested by the ubiquity and non-random distribution of sequences such as (CT)n'(GA)n within the genomes of many organisms [24]. GA repeats have been found in promoter regions of many genes, at recombination hotspots and at replication origins [24]. Homopurine-homopyrimidine sequences could influence transcription through several possible mechanisms. It has been suggested that the tendency of these sequences to adopt alternative DNA conformations such as H-DNA could be used to absorb negative supercoils generated in the wake of RNA polymerase, facilitating DNA unwinding within the transcription bubble [24, 25]. Homopurin-homopyrimidine sequences could also influence chromatin structure by serving as binding sites for regulatory proteins or important chromatin assembly fac-

tors. This possibility has been particularly well studied in the case of the Drosophila hsp26 gene [25]. GAGA binding factor is believed to affect several steps in the process of transcription particularly causing RNA polymerase II pausing in many genes during transcription [25]. This phenomenon may be related to premature termination of gene transcription.

To test the possibility that homopurine-homopyrimidine sequences may influence premature termination of TAS3 gene transcription we inserted the clone St.41 into agrobacterial vector for transient expression. Epidermal cells of *N. benthamiana* leaves were agroinfiltrated with this construct where transcription was driven by 35S promoter. At 4 days post infiltration total RNA was isolated. Three out of 9 clones for transiently expressed *C. articulatus* TAS3-Sen1 had the poly A tail added in the middle position (similar to clone St.33 – Fig. 4). The rest of the clones were terminated at the position typical for cauliflower mosaic virus 35S terminator (data not shown). No clones analogous to St.41 (possessing G/A cluster upsteam of poly (A) were observed for infiltrated leaf areas (data not shown).

```
St.41 – TTCTTGACCTTGTAAGACCTTTTCTCGACCTTGTAAGACCCAAAAATACCTTTGGTGTCTTTTCATTTTTTTGTGTTTTGTATTTTAAATCACTCTCAAGTG
Ca.90 – ------------------------T-----------------------------------------C-----C-------G------------T-------
Ca.91 – ------------------------T-----------------------------------------C-----C-------G------------T-------
St.33 – ----------------------------------------------------------------------------------------------------
Cr.28 – ------------------------T-----------------------------------------------------G--------------------
Cr.29 – ------------------------T-----------------------------------------------------G----- – poly(A)
Cr.31 – ------------------------T---------- poly(A)


ATAATTGTATTTTTATCATTTTTCTTCGTTCCCACCCAACTCGTGTTCTCCTTCCTTGTCTATCCCTTCTGAGCTATTTCCTCTCACAATCAACTAGTCAAGGGTTATGT
-----------------G-----------------------A---------------------------------------T-G----------------##--
----------------G----------- – poly(A)
---------------------------- – poly(A)
---------------------------- – poly(A)

GTTTGTTCCAACTCGGAATTAATTAGTTGGTAGAAACAAGCCCTAATATCTCTTTGTTAGTAAACAAAATGTAACCTTATTTCTTGGTCTCCTTCTACAAAACAAGAGCC
----T------------------G-----------------------A---##############################----------G----------T------

AAAACCTTTCGTCCGTCATCTTTAAAAATGCTCCATATATGATGGTATATTAGAGAGAGAGAGAGAGAGAGAGAGAG – poly(A)
-----------------------------------AG-T---ACGACG---TAG – poly(A)
```

Figure 4. Nucleotide sequences of full-length and premature terminated transcripts of TAS3-Sen1 genes from S. talinoides (St.), C. repens (Cr.) and C. articulatus (Ca.). TAS3 ta-siARF region is shaded. Deleted residues are shown as (#)

It was shown recently that alternative polyadenylation would generate two transcripts from the *Arabidopsis* TAS3a locus (At3g17185), only one of which contains both miR390 binding sites flanking ta-siARF region and would be subject to miR390-mediated processing [26, 27]. Such phenomenon allows for more layers of regulation linked to miRNA and ta-siRNA. For example, the non-protein coding gene IPS1 (INDUCED BY PHOSPHATE STARVATION 1) from Arabidopsis thaliana contains a motif with sequence complementarity to the phosphate (Pi) starvation-induced miRNA miR-399, but the pairing is interrupted by a mismatched loop at the expected miRNA cleavage site [28]. As a result IPS1 RNA is not cleaved but instead sequesters miR-399. Thus, IPS1 overexpression results in increased accumulation of the miR-399 target PHO2 mRNA and, concomitantly, in reduced shoot Pi content. Moreover it was found that over-expression of several additional miRNA decoys with non-cleavable miRNA sites result in a certain degree of specific inactivation of individual members of different miRNA families [29]. In principle, the application of alternative transcripts of Senecio TAS3 genes as decoys for the modulation of miR390 activity could be extended to regulate synthesis of ta-siARF RNAs in different Senecio species by permitting varying degrees of miR390 inactivation by their own functional TAS3 target genes.

1. Chen X. Small RNAs – secrets and surprises of the genome // Plant Journal. – 2010. – Vol. 61, № 6. – P. 941–958. 2. Axtell M. J., Westholm J. O., Lai, E. C. Vive la différence: biogenesis and evolution of microRNAs in plants and animals // Genome Biology. – 2011. – Vol. 12, № 4. – P. 221-234. 3. Xie Z., Khanna K., Ruan S. Expression of microRNAs and its regulation in plants // Semin. Cell. Dev. Biol. – 2010. – Vol. 21, №8. – P. 790-797. 4. Liu Q., Feng Y., Zhu Z. Dicer-like (DCL) proteins in plants // Funct. Integr.Genomics. – 2009. – Vol. 9, №3. – P. 277-286. 5. Xie Z., Qi X. Diverse small RNA-directed silencing pathways in plants // Biochimica et Biophysica Acta. – 2008. – Vol. 1779, №2. – P. 720–724. 6. Allen E., Howell M. D. miRNAs in the biogenesis of trans-acting siRNAs in higher plants // Semin. Cell. Dev. Biol. – 2010. – Vol. 21, №8. – P. 798-804. 7. Chan S. W-L. Inputs and outputs for chromatin-targeted RNAi // Trends in Plant Science. – 2008. – Vol. 13, №7. – P. 383-389. 8. Vaucheret H. MicroRNA-dependent trans-acting siRNA production // Science STKE. – 2005. – Vol. 3. – P. 43-48. 9. Krasnikova M. S., Milyutina I. A., Bobrova V. K., Troitsky A. V., Solovyev A. G., Morozov S. Y. Molecular Diversity of miR390-Guided Transacting siRNA Precursor Genes in Lower Land Plants: Experimental Approach and Bioinformatics Analysis // Sequencing. – 2011. – vol. 2011, Article ID 703683, doi:10.1155/2011/703683. 10. Takeda A., Iwasaki S., Watanabe T. The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins // Plant Cell Physiology. – 2008. – Vol.49, №4. – P. 493-500. 11. Krasnikova M. S., Milyutina I. A., Bobrova V. K., Ozerova L. V., Troitsky A. V., Solovyev A. G., Morozov S. Y. Novel miR390-dependent transacting siRNA Precursors in plants revealed by a PCR-based experimental approach and database analysis // Journal of Biomedicine and Biotechnology. – 2009. – Vol. 2009, Article ID 952304. 12. Yelina N. E, Erokhina T. N. Lukhovitskaya N. I. Localization of Poa semilatent virus cysteine-rich protein in peroxisomes is dispensable for its ability to suppress RNA silencing // J Gen Virol. – 2005. – Vol. 86. – P. 479–489. 13. Piazza P., Jasinski S., Tsiantis M. Evolution of leaf developmental mechanisms // New Phytol. – 2005. – Vol. 167, №3. – P. 693-710. 14. Husbands A.Y., Chitwood D. H., Plavskin Y., Timmermans M. C. Signals and prepatterns: new insights into organ polarity in plants // Genes Dev. – 2009. – Vol. 23, №17. – P. 1986-1997. 15. Yamaguchi T, Yano S, Tsukaya H. Genetic framework for flattened leaf blade formation in unifacial leaves of Juncus prismatocarpus // Plant Cell. – 2010. – Vol. 22, №7. – P. 2141-2155. 16. Garcia D., Collier S. A., Byrne M. E., Martienssen R. A. Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway //Current Biology. – 2006. – Vol. 16. – P. 933–938. 17. Nogueira F. T., Madi S., Chitwood D. H., Juarez M. T, Timmermans M. C. Two small regulatory RNAs establish opposing fates of a developmental axis //Genes & Development. -2007. – Vol. 21. – P. 750–755. 18. Douglas R. N., Wiley D., Sarkar A., Springer N., Timmermans M. C., Scanlon M. J. Ragged seedling2 encodes an ARGONAUTE7-like protein required for mediolateral expansion, but not dorsiventrality, of maize leaves // Plant Cell. – 2010. – Vol. 22, №5. – P. 1441-1451. 19. Ozerova L.V., Timonin A.C. On the evidence of subunifacial and unifacial leaves: Developmental studies in leaf-succulent Senecio L. species (Asteraceae) // Wulfenia. – 2009. – Vol. 16. – P. 61-77. 20. Bettin O., Cornejo C., Edwards P. J., Holderegger R. Phylogeography of the high alpine plant Senecio halleri (Asteraceae) in the European Alps: in situ glacial survival with postglacial stepwise dispersal into peripheral areas // Mol Ecol. – 2007. – Vol. 16, №12. – P. 2517-2524. 21. Duarte J. M., Wall P. K., Edger P.P., Landherr L.L., Ma H., Pires J.C., Leebens-Mack J., de Pamphilis, C.W. Identification of shared single copy nuclear genes in

Arabidopsis, Populus, Vitis and Oryza and their phylogenetic utility across various taxonomic levels // BMC Evol. Biol. – 2010. – Vol. 10. – P. 61-70. 22. Jagadeeswaran G., Zheng Y., Li Y. F., Shukla L.I., Matts J., Hoyt P., Macmil S. L., Wiley G. B., Roe B. A., Zhang W., Sunkar R. Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families // New Phytol. – 2009. – Vol. 184, №1. – P. 85-98. 23. Alexandrov, N.N., Troukhan, M.E., Brover, V.V., Tatarinova, T., Lu, Y.-P., Flavell,R.B., Feldmann,K.A. Features of Arabidopsis genes and genome discovered using full-length cDNAs // Plant Mol. Biol. – 2006. – Vol. 60, №1. – P. 69-85. 24. Quinn L., Teare J. M., Granok H., Swede M. J., Xu J., Elgin S.C.R. The capacity to form H-DNA cannot substitute for GAGA factor binding to a (CT)n'(GA)n regulatory site // Nucleic Acids Research. – 2003. – Vol. 31, No. 10. – P. 2483-2494. 25. Lee C., Li X., Hechmer A., Eisen M., Biggin M. D., Venters B. J., Jiang C., Li J., Pugh B. F., Gilmour D. S. NELF and GAGA Factor Are Linked to Promoter-Proximal Pausing at Many Genes in Drosophila // Mol Cell Biol. – 2008. – Vol. 28, №10. – P. 3290-3300. 26. Hunt A. G. RNA regulatory elements and polyadenylation in plants // Frontiers in Plant Science. Plant Genetics and Genomics. – 2012. – Vol. 2, article 109. 27. Wu X., Liu M., Downie B., Liang C., Ji G., Li Q.Q., Hunt A.G. Genome-wide landscape of polyadenylation in Arabidopsis provides evidence for extensive alternative polyadenylation // Proc Natl Acad Sci U S A. – 2011. – Vol. 108, №30. – P. 12533-12538. 28. Franco-Zorrilla J. M., Valli A., Todesco M., Mateos I., Puga M.I., Rubio-Somoza I., Leyva A., Weigel D., García J. A., Paz-Ares J. Target mimicry provides a new mechanism for regulation of microRNA activity // Nat Genet. – 2007. – Vol. 39, №8. – P. 1033-1037. 29. Ivashuta S., Banks I. R., Wiggins B. E., Zhang Y., Ziegler T. E., Roberts J. K., Heck G. R. Regulation of gene expression in plants through miRNA inactivation // PLoS One. – 2011. – Vo. 6, №6. – e21330.

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GENETIC MONITORING OF CIRCULATING INFLUENZA VIRUSES IN UKRAINE DURING 2010-2011 INFLUENZA SEASON

Результаты филогенетичского анализа украинских изолятов вируса гриппа эпидемического сезона 2010-2011 показали их схожесть с таковыми в мире. Анализ мутаций обнаружил важные аминокислотные замены в генах гемагглютинина украинских изолятов.

The phylogenetic analysis of Ukrainian influenza viruses during 2010-2011 epidemic season showed their similarity to flu isolates all around the world. Mutation finding indicated significant amino acid substitution in the hemagglutinin genes of Ukrainian viruses.

Annual influenza epidemics in many countries, considerably in Northern Hemisphere, are caused by continuous variability of influenza viruses and extreme activity of their transmission. In fact, epidemic season 2010-2011 has lower intensity and les number of selected viruses from the previous 2009-2010 pandemic season.

Monitoring the genetic changes of influenza viruses appeared in our country not long ago, but has already proved its relevance and necessity. Such data can considerable speed up the adequate response to new or changed viruses. So, the work of all public health services in country was adjusted, after confirmating first selected pandemic A(H1N1) sw influenza isolate.

The aim of our study was to perform the genetic monitoring of influenza viruses isolated in Ukraine and to compare with viruses from other countries which circulated during the 2010-2011 epidemic season by constructing the phylogenetic trees.

Materials and methods. Nasopharyngeal swabs and autopsy materials collected from infected patients, were received during the epidemic from different regions of Ukraine. Typing of viruses was performed by real-time RT-PCR (reverse transcription polymerase latsyuhova reaction in real time). Strain definition was performed with using the diagnostic serum sent by Center for Disease Control and Prevention (CDC Atlanta, USA). For genetic analysis were used sequences of hemagglutinin gene of Ukrainian influenza viruses A(H1N1)sw, A(H3N2) and B. The GISAID web resource [1] was used for searching sequences essential for the analysis. The identification and comparison of obtained sequences was performed in BLAST (Basic Local Aligment Search Tool) [2]. Phylogenetic comparison and constructing the phylogenetic trees was performed in MEGA 5 program software [3] with using the Neighbor-Joining (combining nearest neighbors) [4] method. The aligning of sequences was performed with using ClustalW algorithm [5]. For the statistical evaluation of the results significance was used Bootstrap analysis with the number of replications 1000 [6].

Results and discussion. Influenza season 2010-2011 was the first after the pandemic season in Ukraine. The distinction of this season was early wide circulation of influenza B viruses (autumn 2010) and ongoing circulation of pandemic A(H1N1)sw influenza viruses (from the second half of January and until the beginning of April). And only two of A(H3N2) influenza viruses have been isolated. Genetic analysis was directed to hemagglutinin genes as those with high level of variability. More over the hemagglutinin genes are the primary target for neutralizing antibodies.

Phylogenetic comparison of hemagglutinin (HA) genes of influenza Asw (H1N1) viruses

The pandemic A (H1N1) sw influenza viruses continued to be isolated in the 2010-2011 epidemic season despite their appearence in early 2009 by triple gene recombination of human, swine and bird influenza viruses [7]. It is known from the literature that hemagglutinin of pandemic influenza viruses has classical swine flu virus derivation (like ones, caused the pandemic of 1918-1919), which distinguishes them from human or birds influenza viruses [8].

We began to research the hemagglutinin sequences of Ukrainian isolates from finding similar genetic sequences with using BLAST search too. So, we found viruses with high percentages of similarity for the hemagglutinin genes: isolate from a 36 years old man, who has ill after contact with a sick animal – A/Ohio/01/2007 (the similarity 93%), and swine influenza virus – A/swine/Illinois/1/1975 (similarity – 87%). Also in analysis were taken the reference strain of seasonal human influenza virus (H1N1) – A/New Caledonia/20/1999 with 7% similarity (Fig. 1.).

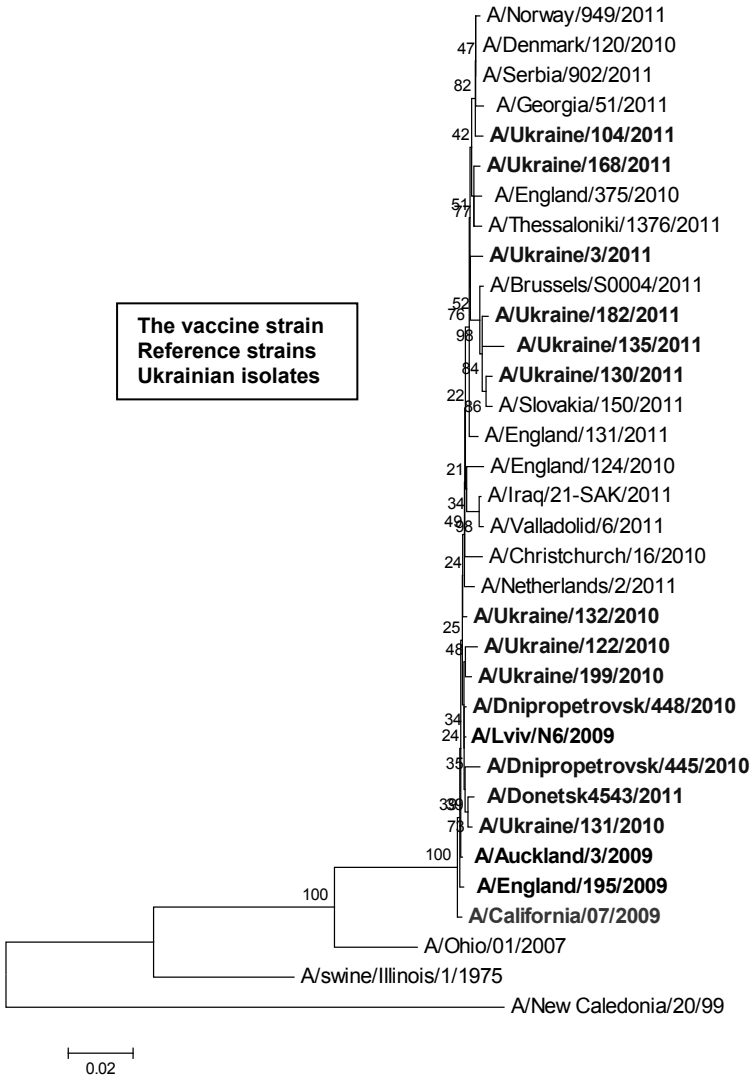

Fig.1. Phylogenetic tree of hemagglutinin (HA) gene sequences of influenza A(H1N1)viruses constructed by NJ method with Kimura 2-parameter model (Bootstrap values are indicated)

According to the results of Hemagglutinin inhibition (HI) assay our isolate A/Lviv/N6/2009 was selected as reference strain due to its low binding activity to ferret antisera comparing to the vaccine strain A/California/07/2009 (in titer of 160 against 1280).

Having completed all steps of analysis, we obtained phylogenetic tree where hemagglutinin genes of Ukrainian isolates comparably to viruses isolated in other countries, including vaccine and reference strains (Fig. 1.). We can see from the figure that viruses isolated in Ukraine in autumn 2010 placed at the bottom of phylogenetic tree near the reference strain A/Lviv/N6/2009. But isolates fron early 2011 had gained changes that determined them in another cluster. Also we have found one exception – A/Donetsk/4543/2011, which remained genetically similar to isolates from autumn 2010, although was isolated in 2011.

Also, for more detailed analysis of the hemagglutinin gene of pandemic influenza viruses, we constructed a phylogenetic tree, with only pandemic influenza viruses (Fig. 2.). More over, we find amino acid differences in sequences of hemagglutinin proteins of Ukrainian isolates in comparison with the vaccine and reference strains. Three isolates, A/Ukraine/130/2011, A/Ukraine/135/2011 and A/Ukraine/182/2011 formed a separate cluster with viruses isolated in Brussels and Slovakia according their differences. In hemagglutinin of these viruses were found following amino acid substitutions: S143G (glutamine changed serine at position 143) and A197T (threonine changed alanine at position 197).

In addition, isolate A/Ukraine/135/2011 showed polymorphism in the nucleotide sequence encoding the 155 amino acid residue of hemagglutinin protein. According to the World Influenza Centre (London) polymorphism or nucleotide substitution in the region, which encodes residues from 153 to 157 is often associated with low binding ability to antisera. It is interesting that the polymorphism in this region was not observed in clinical samples. It can be an artifact of reproduction in cell culture, especially for the MDCK-siat line, that ceased to use in study of influenza A(H1N1)sw viruses [9].