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Date received: 15/10/2016
Date reviewed: 9/11/2016
Reviewer: Dr. Khuat Huu Trung
Date approved for publication: 20/12/2016

INSIGHT INTO THE *NUCLEAR FACTOR-YA* TRANSCRIPTION FACTOR FAMILY IN SWEET ORANGE (*Citrus sinensis*)

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Abstract

Nuclear factor Y (NF-Y), including NF-YA, NF-YB and NF-YC is well-established to play an important role in regulation of the developmental processes and stress responses in plants. In this study, 6 members of the *NF-YA* gene family were identified in the sweet orange (*Citrus sinensis*) genome. Among them, 2 members, CsNF-YA1 and CsNF-YA6 might be localized in the chloroplast, while CaNF-YA3 was thought to be associated with the secretary pathway. The common motif of the exon/intron organization of the *CaNF-YA* contains 5 exons and 4 introns. The conserved region of NF-YA is characterized by 2 typical domains, including the interaction NF-YB/C domain and the DNA binding domain. Next, various *cis*- regulatory elements related to the light responsiveness, tissues-specific expression and hormonal/stress responsiveness were predicted to be distributed on the promoter regions of *NF-YA* family, suggesting that this gene family might be related to the light conditions, tissue-specific expression and response to hormonal/stresses. Based on the available RNA-Seq, all of *CsNF-YA* genes were up-regulated in at least 1 organ/tissue. Interestingly, two genes, *CsNF-YA1* and *CsNF-YA6* were specific in callus, while *CsNF-YA4* was preferentially expressed in fruit. Three remaining genes were highly expressed in all 4 tissues/organs, including callus, flower, fruit and leaf.

Keywords: *Citrus sinensis,* NF-YA, *in silico,* sweet orange, transcription factor, stress

INTRODUCTION

NF-YA (Nuclear factor Y), one of the most important transcription factor (TF) family, was likely found in all eukaryotes. Based on the binding to the CCAAT elements, TF NF-Y proteins have roles in the regulation of diverse genes, therefore be related to various biological processes, such as the signaling pathway, and in plant stress responses (Laloum *et al*., 2013). Generally, NF-YA, including 3 subunits - NF-YA, NF-YB and NF-YC, of which NF-YA, with DNA binding sites and interaction NF-YB/C, is known as the most important subunit. Therefore, study on the genes encoding *NF-YA* is one of the interests that could be provide an insight into the regulation of NF-Y family in cellular biological processes, especially in the defense mechanisms of plant cells against the adverse environmental conditions.

Up to now, taking the advantages of omics era, NF-YA gene families have been identified and characterized in many plant species, such as in the model plant *Arabidopsis thaliana* (Siefers *et al*., 2009), *Brachypodium distachyon* (Cao *et al*., 2011), and several important crops - rice (*Oryza sativa*) (Thirumurugan *et al*., 2008), canola (*Canola napus*) (Liang *et al*., 2014), soybean (*Glycine max*) (Quach *et al*., 2015), foxtail millet (*Setaria italica*) (Feng *et al*., 2015). More recently, *NF-YA* gene family was also discovered in several newly annotated plant's

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genomes, such as tomato (*Solanum lycopersicum*) (Li *et al*., 2016), grape (*Vitis vinifera*)(Ren *et al*., 2016), sorghum (*Sorghum bicolor*) (Malviya *et al*., 2016). But no information was reported about the *NF-YA* gene family in sweet orange (*Citrus sinensis*), one of the most important sweet fruit plant grown in the tropical areas throughout the world and in Vietnam.

In this study, the general information of *NF-YA* gene family in sweet orange was firstly provided. Nomenclature, gene annotation and several typical characteristics of *NF-YA* genes of sweet orange were identified by using various bioinformatics approaches. Gene organization, conserved domain structure were then harvested to evaluate the diversity and the preservation of NF-YA family. Next, the prediction of the presences of *cis*- regulatory elements in the promoter regions of *NF-YA* gene family were carried out in order to get a first glance into the roles of *NF-YA* genes in the regulation of the biological processes and stress responses of plant. Finally, the expression profiles of *NF-YA* genes in various tissues/organs were retrived using the available RNA-seq data.

MATERIALS AND METHODS

Materials

- The sweet orange genome Valencia database (Xu *et al*., 2013) available in the PHYTOZOME v11.0.

Methods

Insilico identification of the NF-YA *gene family in the sweet orange genome*

- To identify all members of *NF-YA* family in sweet orange, a typical protein containing *CBFB_NFYA* conserved domain (Pfam, PF02045) (Laloum *et al*., 2013) was used as a seed sequence to BLASTp against the sweet orange genome (Xu *et al*., 2013) (E-value < 1×10^{-6}) in Phytozome database.

- The nomenclature and annotation of *NF-YA* gene family were collected based on the NCBI database (Bioproject: PRJNA86123) (Xu *et al*., 2013).

- The general information, including the isoelectric point (pI), molecular weight (mW), was collected through the Expasy tool. The subcellular localization of proteins was predicted via the TargetP v1.1 web-based tool.

Gene structure analysis of the NF-YA *gene family in sweet orange*

- The Gene Structure Display Server (GSDS) v2.0 was used to analyze the exon/intron organization of the *NF-YA* gene family in cassava by using the CDS (Coding DNA sequence) of each gene.

- The promoter regions (1kb upstream from the start codon site) of all *NF-YA* genes were obtained from Phytozome database. The presence of *cis*- regulatory elements was determined through PlantCARE web server as previously reviewed (Chu and Le, 2015).

Expression analysis of the NF-YA *gene family*

- To analyze the transcriptome data for the identified *NF-YA* genes of sweet orange, the protein sequence of each gene was BLASTp against the CAP database v2.1 (*Citrus sinensis* Annotation Project) (Xu *et al*., 2013). The expression profiles of *NF-YA* genes in various tissues/organs were retrieved based on the previous RNA-Seq result (Jiao *et al*., 2013).

RESULTS AND DISCUSSION

Genome-wide identification of the *NF-YA* gene family in the sweet orange genome

In order to identify the *NF-YA* gene family in sweet orange, a comprehensive BLASTp search of protein containing typical NF-YA conserved domain (Laloum *et al*., 2013) was performed against the Phytozome database. As a result, a total of 6 members of the *NF-YA* family have been found in the sweet orange genome (E-value < 1×10^{-6}). The gene annotation and nomenclature of *NF-YA* gene family were harvested by searching against the NCBI database (Bioproject: PRJNA86123) (Xu *et al*., 2013) (Table 1).

Table 1. Annotation of *NF-Y* gene family in sweet orange genome

#	Gene name	Gene code[1]	Protein code[1]	Locus name[2]	Gene symbol[1]
1	*CsNF-YA1*	XM_006465894	XP_006465957	orange1.1g019782	LOC102611325
2	*CsNF-YA2*	XM_006470556	XP_006470619	orange1.1g019036	LOC102628969
3	*CsNF-YA3*	XM_006468242	XP_006468305	orange1.1g026474	LOC102608084
4	*CsNF-YA4*	XM_006481552	XP_006481615	orange1.1g021081	LOC102630159
5	*CsNF-YA5*	XM_006482808	XP_006482871	orange1.1g019764	LOC102616312
6	*CsNF-YA6*	XM_006489857	XP_006489920	orange1.1g017825	LOC102620218

Note: Information were collected from [1]NCBI and [2]Phytozome database.

As provided in the table 2, six members of *NF-YA* gene family were distributed in the chromosomes with an uneven ratio. Among them, two genes, *XM_006470556* and *XM_006468242* were located in the chromosome 2, whereas the remaining genes were distributed in the chromosome 1, 6, 7 and 9, respectively. As this work was the first comprehensive identification and characterization study of the *CsNF-YA* family in sweet orange, we suggested a universal nomenclature for 6 members, following the chromosomal order. In this study, the identification of 6 *CsNF-YA* genes clearly revealed that TF NF-YA family in sweet orange was also a multigene family as reported in other plant species, such as in common bean (9 *PvNF-YA* genes) (Ripodas *et al*., 2014), soybean (21 *GmNF-YA* genes) (Quach *et al*., 2015), tomato (10 *Solyc* genes) (Li *et al*., 2016), rice (10 *OsHAP2* genes) (Thirumurugan *et al*., 2008), sorghum (8 *SbNF-YA* genes) (Malviya *et al*., 2016), grape (8 *VvNF-YA* genes) (Ren *et al*., 2016). Our findings, taken together, strongly confirmed that *NF-YA* gene family was variously amplified in plant species, and the difference of chromosomes in each plant's genome may cause the diversity of genes encoding NF-YA subunit.

Table 2. General information of NF-YA family in sweet orange

#	Gene name	Chromosomal location[1]	L[1]	mW[2]	pI[2]	SL[3]
1	*CsNF-YA1*	Chr1F (20916865..20921068)	336	36,67	8,67	C*
2	*CsNF-YA2*	Chr2F (29713691..29718461)	347	37,26	6,26	-
3	*CsNF-YA3*	Chr2R (7234359..7239176)	238	26,52	9,15	S*
4	*CsNF-YA4*	Chr6F (14983555..14988520)	317	34,64	9,27	-
5	*CsNF-YA5*	Chr7R (461421..464375)	336	36,77	9,54	-
6	*CsNF-YA6*	Chr9F (10756754..10763202)	365	39,87	9,44	C*

*Note: Information were collected from [1]NCBI, [2]Expasy and [3]TargetP tools; Chr - Chromosome; F - Forward; R - Reverse; L - Length (amino acid); mW - Molecular weight (kDa); pI - Isoelectric point; SL - Subcellular localization; S - Secretory pathway; C - Chloroplast; "-" - Any other location; * - Reliable prediction.*

In this study, several typical characteristics of CsNF-YA family of sweet orange were also analyzed by querying the protein sequence of each member in various bioinformatics tools. Firstly, the protein sizes of all members of NF-YA family in sweet orange were varied from 238 (CsNF-YA3, XP_006468305) to 365 amino acid (CsNF-YA6, XP_006489920). Relevant for the molecular weight, they were approximately calculated from 26.52 to 39.87 kDa. Next, most of CsNF-YA were basic proteins, the pI values were ranged from 8.67 to 9.54, except for CsNF-YA2 (pI = 6.26) (Table 2). The relationship between pI values and subcellular localization of proteins were previously well-established, therefore might suggest the function of the predicted proteins (Kiraga *et al*., 2007). Here, most of members of CsNF-YA might be targeted in the membrane, perhaps in the membrane of the organelles. To further enhance the reliability of prediction, TargetP tool was used to identify the subcellular localization of CsNF-YA family.

TargetP was then used to predict the subcellular localization of CsNF-YA based on the determination of the signal peptide as previously reported. As a result, the localization of 3 CsNF-YA proteins were predicted with high reliable scores. Among them, CsNF-YA1 and CsNF-YA6 were identified to distribute into the chloroplast, while CsNF-YA3 might be located in the secretory pathway. It is well-known that chloroplast, one of the most important organelles, is the site of high accumulation of ROS under the effects of adverse environmental conditions. We raised a question about whether CsNF-YA1 and CsNF-YA6 were involved in the defense mechanism in the chloroplast or not and CsNF-YA3 were contributed in the regulation of the biological processes via the signal pathway? Recently, the role of NF-YA were characterized to be involved in the growth, development of plants (Li *et al*., 2016), especially in response to the abiotic stresses (Quach *et al*., 2015).

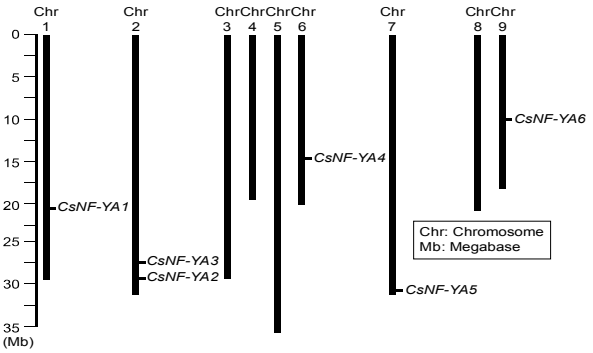

Figure 1. The chromosomal location of *NF-YA* family in the sweet orange genome

Results on analysis of the conserved domain and gene structure of NF-YA family in sweet orange

To get insight into the diversity of gene structure of *NF-YA* gene family in sweet orange, the exon/intron organization were harvested based on the CDS sequence of each member. The gene structure were analyzed by using the GSDS 2.0 web-based tool (Hu *et al.*, 2015) and arranged as following the order of the phylogenetic tree (Figure 2).

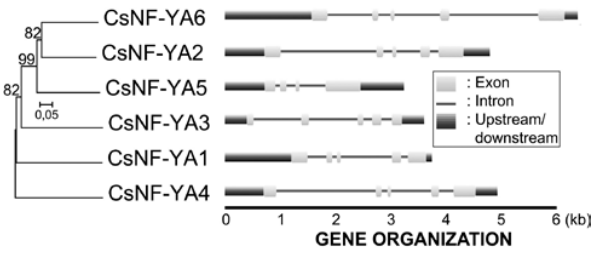

Figure 2. Exon/Intron organization of the *NF-YA* family of sweet orange

As described in the figure 2, the common motif of exon/intron organization of *NF-YA* gene family was found to be 5 exon/4 intron, except for *CsNF-YA5* (4 exon/3 intron). It is noticeable that the introns are well-established to be the critical entities of the eukaryotic genes. The gain and loss of the introns in the CDS region of a gene causes the structural diversity and complexity, which might be related to the evolution of multiple gene families like *NF-YA* gene family.

Next, the full-length proteins were used to analyze and evaluate the conserved domain of NF-YA family of sweet orange. As a result, the conserved domain of NF-YA family can be characterized by 2 typical domains, including the interaction NF-YB/C domain and the DNA binding domain, as relevant to other plant species (Laloum *et al.*, 2013). Our findings strongly suggested that the purifying selection was appeared to maintain the preservation of NF-YA protein as is, and the roles of NF-YA were protected during the time selection of the evolution. To further characterize the role of *NF-YA* gene family in sweet orange, the presence of *cis*- regulatory elements in the promoter regions were carried out.

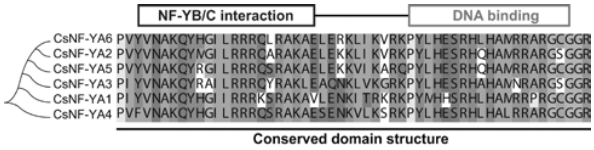

Figure 3. The conserved domain of NF-YA family in sweet orange

The prediction of the *cis*- regulatory elements in the promoter regions of the *NF-YA* genes in sweet orange

In order to get insight into the function and the regulation of *NF-YA* family in sweet orange, the *cis*- regulatory elements were determined in the promoter (1000-bp-upstream regions from the start codon site) of each gene. Several basic elements, such as CAAT-box and TATA-box were found in all promoter regions of *CsNF-YA* genes. Many regulatory elements were also identified in these promoter regions. Obviously, these *cis*- elements can be classified into 4 functional groups, including (i) light responsiveness, (ii) tissues-expression specific, (iii) hormonal responsiveness and (iv) stress responsive elements. Our data suggested that, *NF-YA* family in sweet orange might be involved in the light condition responsiveness, and the expression of *NF-YA* genes were noticed to be specific in some important tissues/organs.

Table 3. The presences of several well-established hormonal/stress responsive cis- regulatory elements in the promoter regions of *NF-YA* gene family in sweet orange

Promoter	LTRE	MBS	TC-rich repeats	HSE	ABRE	ERE	GARE	SaRE	MeJARE
CsNF-YA1			Y		Y		Y		
CsNF-YA2				Y			Y		
CsNF-YA3	Y	Y		Y		Y			
CsNF-YA4	Y	Y	Y	Y			Y	Y	
CsNF-YA5								Y	Y
CsNF-YA6		Y				Y	Y		Y

Note: LTRE: Low Temperature Responsive Element; MBS: MYB Binding Site; HSE: Heat Stress Element; ABRE: Abscisic acid Responsive Element; ERE: Ethylene Responsive Elements; GARE: Gibberellin Responsive Elements; SaRE: Salicylic Acid Responsive Elements; MeJARE: Methyl Jasmonate Acid Responsive Elements; Y: The presence of cis- regulatory element.

As shown in Table 2, all promoters of *CsNF-YA* gene family contained at least one type of elements related to the hormonal responsiveness. Here, abscisic acid (ABA), ethylene, gibberellin (GA), salicylic acid (SA) and methyl jasmonate acid (MeJA) are some of the most important hormones were focused on this analysis. Among them, regulatory elements related to GA and ethylene were found to be distributed on the promoter of *CsNF-YA2* and *CsNF-YA3* genes, respectively, whereas promoter region of *CsNF-YA6* gene contained the *cis*- elements related to 3 hormones, including MeJA, GA and ethylene. Basically, the presences of several elements associated with *MeJA* pathway in the promoter regions of *CaNF-YA5* and *CaNF-YA6* genes suggested that these 2 genes may be involved in the response to biotic stress of plants. Additionally, the distribution of ABRE in the promoter of *CsNF-YA1* raised a question that this gene might be contributed on the defense mechanism to adverse environmental conditions via the ABA-dependent pathway or not (Chu and Le, 2015)?

To our interest, several stress- responsive elements were also found in the promoters of *NF-YA* genes in sweet orange. Some well-known stress related elements were the low temperature responsive element (LTRE), MYB binding site (MYB) related to drought/salt stress, TC-rich repeats associated with defense mechanism and stress responsiveness and heat stress element (HSE) (Chu and Le, 2015). No stress related elements were recorded in the promoter of *CsNF-YA5* gene, whereas promoter of *CaNF-YA4* contained all mentioned-above *cis*- elements. Additionally, TC-rich repeats, HSE and MBS were distributed in the promoter regions of 3 genes, *CaNF-YA1, CaNF-YA2* and *CaNF-YA6*, respectively.

To sum up, the presences of various *cis*- regulatory elements in the promoter regions of *NF-YA* gene family in sweet orange indicated that these *NF-YA* genes may be associated with the hormonal signaling pathway, and perhaps in the response to the abiotic stresses. Furthermore, their promoter could be used in genetic engineering of sweet orange plants against the adverse environmental conditions.

Expression profiles of *CsNF-YA* genes in various tissues, organs

To get an insight into the functional attributes of *NF-YA* genes of sweet orange, an effort has been made to analyze expression profiles using available transcriptome data of sweet orange in various tissues/organs (Jiao *et al*., 2013). The CAP database provided a comprehensive transcriptome atlas that was generalized for 4 major tissues/organs of sweet orange plant, including callus, fruit, flower and leaf, using RNA sequencing (Jiao *et al*., 2013). The transcript level of each gene was approximately calculated by RPKM values (reads per kilobase per million-mapped reads). Overall, the expression data for *NF-YA* gene family in these tissues/organs were presented in a heat map shown in Figure 4.

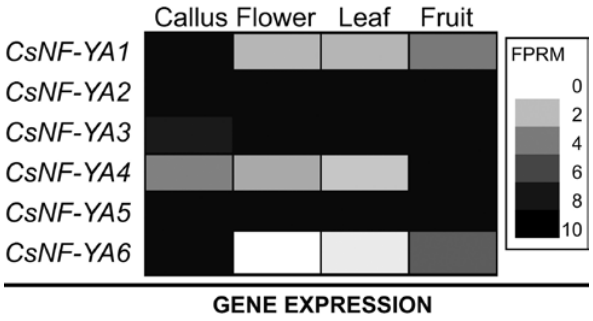

Figure 4. Expression profiles of *NF-YA* gene family in various tissues and organs

According to the FPRM values retrieved from the available RNA-seq data (Jiao *et al*., 2013), all *CsNF-YA* genes were expressed at high level in at least 1 organ/tissue. Interestingly, two genes, *CsNF-YA1* and *CsNF-YA6* seemed to be exclusively specific in callus, and *CsNF-YA4* were strongly induced in fruit. Other genes were noticed to distribute in whole tissues/organs. As expected, the high accumulation of the transcript of *NF-YA* gene family in these important tissues/organs, suggesting that NF-YA family might be involved in various processes in sweet orange plant, such as be related to the growth and development stages, the fruit ripening, and perhaps in the defense mechanisms of plant against the abiotic stresses.

CONCLUSION

- Six members of *NF-YA* gene family were identified in the sweet orange genome. These *NF-YA* genes were distributed in the chromosomes with an uneven ratio. CsNF-YA1 and CsNF-YA6 might be located in the chloroplast, while CsNF-YA3 seems to be distributed in the secretory pathway.

- The common motif of gene structure of *NF-YA* genes in sweet orange was 5 exons/4 introns. The different number of introns in the CDS regions of *NF-YA* gene family clearly indicated the diversity of *NF-YA* genes in not only sweet orange but also other plant species. The conserved domain of NF-YA proteins can be characterized by 2 distinct domains, including the interaction NF-YB/YC domain and DNA binding domain.

- Various *cis*- regulatory elements related to the light responsiveness, tissues-specific expression, hormonal/stress responsiveness were found in the promoter regions of *NF-YA* genes in sweet orange. The presence of least 1 group of hormonal/stress responsive elements in the promoters of *NF-YA* gene family suggested that these *NF-YA* genes might be involved in the signaling pathway and associated with the response to the adverse environmental conditions.

- Our expression analysis indicated that all *CsNF-YA* genes seemed to be highly expressed in at least 1 major organs/ tissues. Among them, two genes, *CsNF-YA1* and *CsNF-YA6* were exclusively induced in callus, while *CsNF-YA4* was specific in fruit. Three remaining genes were referred to be specifically expressed in all major tissues/ organs.

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Date received: 21/10/2016
Date reviewed: 25/11/2016
Reviewer: Dr. Tran Ngoc Thanh
Date approved for publication: 20/12/2016

PRIMARY EVALUATION OF SOME TEMPERATE FRUIT CULTIVARS INTRODUCED FROM TAIWAN

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Abstract

14 cultivars of 3 temperate fruits including 4 persimmon cultivars, 4 peach cultivars and 6 pear ones with low and medium chilling requirement introduced from Taiwan were primarily evaluated in 4 Northern Mountainous provinces and Lam Dong (Central Highlands) for its adaptability in Vietnam conditions and from which highly adapted promising cultivars should be screened for large scale production. After more than 3 years of study, some local rootstock cultivars having good compatibility with introduced ones were determined. In addition, introduced cultivars, named Mackawa Jiro (persimmon), B112 and A2-2-39 (peach), Mi Xue and Heng Shan (pear) were considered to be promising in terms of healthy growth, adaptable yield and high quality.

Keywords: Temperate fruits, Chilling requirement, adaptability

INTRODUCTION

Taiwan agricultural varieties in general and fruit ones in particular have good potential in productivity and quality as well as partly contributed to Vietnam agricultural production in past decades, indeed (Le Duc Khanh *et al.*, 2007; P. Blanchet, J. Bourdeaut, Ha Minh Trung, Le Duc Khanh. Dang Vu Thi Than *et al.*, 2000). Under a framework of planting material exchange between Taiwan Agricultural Research Institute (TARI) and Vietnam Academy of Agricultural Sciences (VAAS), 14 cultivars of 3 Taiwan temperate fruits were primarily evaluated at 6 local sites located in 4 Northern Mountainous provinces (Son La, Lao Cai, Bac Kan and Lang Son) and 1 Central Highland province (Lam Dong). Results conducted from the study were summarized in this paper.

MATERIALS AND METHODS

Materials

14 fruit varieties introduced from Taiwan of low to medium chilling requirement (250 - 450 CU) including 4 peach cultivars named B115; A2-2-39; Flordared and Tropic Beauty, 4 persimmon cultivars named Hiratone Nashi, Tone Wase, Nishimura Wase and Mackawa Jiro, 6 pear ones named Mixue, Ming Fu, Heng Shan, Jinxian, Zhizin and Gao Qiang were used for the study. Seedling rootstock varieties (sown in nurseries) are indigenous ones that have good growth and high resistance to main insects and diseases whereas both local and introduced cultivars (double grafting) were used as rootstock ones for Top-working implementation.

Methods

- The layouts of trials concerned were accordingly designed depending on purpose and particularity of the study in which RCBD applied for perennial fruits to make sure all treatments in one repeat having quite similar condition was prioritized (.R.A.I Drew, Ha Minh Trung, Le Duc Khanh 2001; A.P. George, R.J. Nissen, 1998).

- The percentage of grafted shoot survival was used as the indicators of compatibility of introduced varieties when grafted on rootstock seedlings in nurseries or top-worked on aged fruit trees in the orchards (P. Blanchet, J. Bourdeaut, Hà Minh Trung, Le Duc Khanh. Dang Vu Thi Than *et al*. 2000). This combines with the growth of grafted shoots presents the adaptability of fruit varieties introduced in different locations.

- Flowering process, fruit setting, productivity and fruit quality of introduced cualtivars were evaluated and calculated through the samples randomly taken from orchards in combination with bio chemical analysis in the laboratories.

- Collected data were then statistically treated by EXCEL program.

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